The Role of Vascular Endothelial Growth Factor and other cytokines in the aetiology of heavy menstrual bleeding in women with uterine fibroids.

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Abstract

Introduction:

The human endometrium undergoes cyclical changes of proliferation, differentiation and shedding. This cyclical process has been described as an inflammatory process. Menstrual abnormalities account for the morbidity of a large population of females in their reproductive age. Aberration in endometrial angiogenesis has been implicated in the mechanism of heavy menstrual bleeding (HMB). Although the precise mechanism for control the endometrial neoangionesis is not fully understood, vascular endothelial growth factor and other cytokines such as cyclooxygenases, prostaglandins, interleukin -8 and leukocytes have been implicated in both endometrial pathologies and angiogenesis dysregulation. In addition, heavy menstrual bleeding results from upregulation of the expression/synthesis of these local markers.

Uterine fibroids are the most common benign tumor affecting the female reproductive tract. Heavy menstrual bleeding is the main presenting complaint of women with uterine fibroids. However, the mechanism by which uterine fibroids cause heavy menstrual bleeding has not been elicited yet. Therefore, the mechanism of action of different available treatments for this condition, including uterine artery embolisation is unclear. This thesis is based on the hypothesis that a) uterine fibroid changes the physiology of endometrium and we aimed to find out whether these markers work in a different way in heavy menstrual bleeding in those with uterine fibroids and those without., In addition I we wished to study whether uterine fibroid upregulate these local markers in heavy menstrual bleeding, whereas uterine artery emolisation down-regulates them.

Methods:

This thesis describes the use of endometrial samples, taken with a Pipelle sampler, collected from women with heavy menstrual bleeding both with uterine fibroids and also with normal uteri, to estimate the difference in the

endometrial expression of the factors likely to be involved in the control of menstrual bleeding between the two groups.

Results:

The study found no differences between the expression of both either proteins or mRNA for the cytokines under investigation By using endometrium, myometrium and different types of fibroid tissue collected from women who had hysterectomies with the complaint of heavy menstrual bleeding, there was higher expression of VEGF, COX-2, PGE2 and IL8 proteins in fibroid than myometrial tissue. However, the level mRNA of expression for VEGF, COX-1, COX-2, IL8 and EP2 showed no differences between myometrial and fibroid tissue.

In the same group, endometrial expression of these markers for women who had no hormonal therapy before operation compared with that for women who received gonadotropin releasing hormone agonists (GnRH), higher expression of VEGF mRNA in women who had GnRH agonists than those who had no any hormones. In fibroid tissue, GnRH downregulated the expression of VEGF protein and other cytokines compared with those not on any hormonal therapy. In addition, the estimated serum levels of these factors, indicating a higher level of IL8 in the GnRH group than in the other group.

Conclusion:

It seems that theses markers play a role in HMB mechanism in both uterine fibroid and normal uteri group in same manner. In addition, they have a fundamental role in the growth of uterine fibroids as well.

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Author’s Declaration

Except where due acknowledgment is made by reference, the studies undertaken in this thesis were unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted for another degree or professional qualification.

Dr Salha Ali MuamerAbukhnjr

Definitions/Abbreviations

11HSD-1	Hydroxylsteriod dehydrogenase-1
AA	Arachidonic acid
APGAR	Activity, Pulse, Grimace, Appearance, and Respiration
bFGF	Basic fibroblast growth factor
cAMP	Cyclic adenosine monophosphate
cDNA	complementary DNA
CNS	Central nervoius system
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
COX-3	Cyclooxygenase-3
CRP	C-reactive protien
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DPX	Dinbutyl phthalate- xylene
E2	Estradiol
E2R	Estrogen receptor
EGF	Epidermal growth factor
EP	Prostaglandin E2 receptor
ERα	Estrogen receptor alpha
ERß	Estrogen receptor beta
EtOH	Ethanol alcohol
FGF	Fibroblast growth factor
FP	Prostaglandin F2 alpha receptor
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehhde-3-phosphate dehydrogenase
GnRH	Gonadotrophin releasing hormone
GR	Glucocorticoid receptors
GRP-α	Growth-related protein-alpha
H_2O_2	Hydrogen Peroxide
HBEGF	Heparin binding epidermal growth factor
HIER	Heat-induced epitope retrieval
HIF-1	hypoxia inducable factor-1
HMB	Heavy menstrual bleeding
ICAM-1.	Intercellular adhesion molecule 1
IgG	Immunoglobulin-G

IHC	Immunohistochemistry
IL15	Interleukin 15
IL1β	Interleukin-1 beta
IL8	Interleukin-8
IL8-1 (CXCR1)	Interleukin-8 receptor-1
IL8-2 (CXCR2)	Interleukin-8 receptor-2
INF-γ	Interferon gamma
LH	Luteinizing hormone
LHRH	Luteinising-hormone releasing hormone
MCP-1	Monocyte chemoattractant protein-1
MMPs	Matrix metalloproteinases
MRgFUS	Magnetic-resonance-guided focused ultrasound surgery
MRI	Magnetic resonance imaging
mRNA	Messanger Ribonucleic acid
MRT	Multiscribe reverse transcriptase
NE	Neutriphilelastase
NK	Neutral killer
NSAIDs	Non-steroidal anti-inflammatory drugs
PAI	Plasminogen activator inhibitor
PBAC	Pictorial Blood Assessment Chart
PDGF	Platelet-derived growth factor
PG2α	Prostaglandin 2 alpha
PGD	Prostaglandin D
PGDS	prostaglandin-D-synthase
PGE2	Prostaglandin E2
PGES	Prostaglandin-E-synthase
PGFS	Prostaglandin-F-synthase
PGG2	Prostaglandin G2
PGH2	Prostaglandin H_2
PGI2	Prostacyclin
PIER	Proteolytic-induced epitope retrieval
PR	Progesterone receptor
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RT	Reverse transcriptase
RTKs	Receptor tyrosine kinases
SD	Standard Deviation
SPRMs	Selective progesterone receptor modulators
TGF-β	Transforming growth factor beta
TNFα	Tumour necrotising factor-alpha

TNF-α	Tumour necrosis factor-alpha
TV USS	Transvaginal ultrasound scan
TXA2	Thromboxane A2
TXS	Thromboxane synthase
UAE	Uterine artery embolisation
uNK	uterine natural killer
VEGF	Vascular endothelial growth factor
VEGF-R1/	Vascular endothelial growth factor- receptor 1
VEGF-R2/KDR	Vascular endothelial growth factor- receptor 2

Chapter 1:

1. Introduction and literature review

1.1 The human uterus and endometrium

The uterus is an essential for reproduction. It is a near pear shaped fibromuscular organ composed of a smooth muscle outer layer and uterine cavity which is lined by the endometrium. The uterus consists of the body of the uterus and uterine cervix. The smooth muscle cells of the uterine wall are steroid responsive, undergoing hypertrophic and hyperplastic changes during a woman's reproductive life. Pre-puberty, the body of the uterus is nearly equal to the cervix in size. However, it can double its size under the influence of oestrogen during puberty. The hyperplastic and hypertrophic capabilities of smooth muscle cells of the uterus, which are influenced by placental steroids, allow the size of the body of the uterus to increase in line with growth of the developing fetus.

The endometrium lines the uterine cavity (body of the uterus) and it is a dynamic tissue undergoing cyclical changes, involving regeneration and remodelling to prepare for possible implantation. These cyclical changes occur throughout the menstrual cycle and are under the influence of the ovarian steroids hormones. Menstruation, which is the bleeding period, signals the end of one cycle and the beginning of the next. Following the menstrual phase (menstruation), the estradiol level rise in blood leading to growth and proliferation of the endometrium and this time represents the proliferative phase of the menstrual cycle. After ovulation, the corpus luteum produces progesterone, the dominant hormone during this phase (secretory phase). Progesterone in association with estradiol prepares the endometrium for possible implantation. In the absence of pregnancy, the corpus luteum regresses leading to decline in the sex steroids hormones levels and triggering mechanism of menstruation.

1.2 Endometrial morphology

1.1.1 Endometrial structure

The endometrium consists of a superficial functional layer, which is shed during menstruation, and deep basal layer from which endometrial regeneration take place.

1.2.1.1 Proliferative phase

The proliferative phase is the pre-ovulatory phase of the menstrual cycle. All tissue components of endometrium during this stage display signs of proliferation which peak on cycle days 8-10 [1]. The hallmarks of proliferation changes are increased mitotic activity in epithelial and stromal cells, and nuclear Deoxyribonucleic acid (DNA) and cytoplasmic Ribonucleic acid (RNA) synthesis. During this phase the surface epithelial regenerates and epithelial glands gradually elongate and curve. The stroma is dense, containing spindle shape cells and stromal oedema tends to regress through the proliferative phase. Before ovulation, during the late proliferative phase, the surface epithelium becomes more swollen and glands become more tortuous. The stroma by this stage is moderately dense and actively growing. These proliferative alternations are more prominent in the functionalis layer than the basalis layer. The biologic rationale for the environmental variation in proliferative indices may lie in the different physiologic functions of the functionalis versus the basalis layer. Where the functionalis is the seat of blastocyst implantation, the basalis layer provides the origin for regeneration of the endometrium following the menstrual shedding of the functionalis layer. Although estradiol (E2) is the prominent sex hormone in the pre-ovulatory period, many authors have demonstrated that intracellular oestrogen receptor (E_2R) and progesterone receptor (PR) concentrations are highest during the pre-ovulatory period of the menstrual cycle[2-7], confirming the hypothesis that PR synthesis is mainly induced by E_2 in the target cells via the E_2-receptor complex mechanism. Therefore, the presence of PR in the proliferative phase is a good evidence of endometrial E_2 sensitivity.

1.2.1.2 Secretory phase

After ovulation, the E_2-primed endometrium undergoes secretory differentiation which is dominated by the action of progesterone. The daily changes during the secretory phase are specific to post-ovulatory endometrium. These alternations are useful for dating the endometrium and to determine whether ovulation has taken place. Because of widely physiological changing, secretory phase can be divided into three stages; early, mid and late secretory.

1.2.1.2.1 Early secretory:

During this stage the epithelial gland cells acquire sub-nuclear intracytoplasmic glycogen –rich vacuolation and the nuclei lose their pseudostratification configuration. Additionally, there is increase in gland diameter and tortuosity.

1.2.1.2.2 Mid-secretory stage

This stage represents the post-ovulatory days 5-9 and the intra-glandular secretion reaches the maximum levels during this period and coincides with the time of blastocyst implantation. In addition, because of the transudation of plasma from circulating blood in endometrial mucosa, this time of the menstrual cycle is characterised by marked stromal oedema. This alternation may be mediated by prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and coincide also with rising E_2 and P levels. Toward the end of this stage the spiral arterioles become much more prominent.

1.2.1.2.3 Late secretory stage

This is the period from post-ovulary day 10 to menses, where predecidualization takes place and consists of cytonuclear enlargement with mitotic activity, and formation of pericellularlaminin rich basement membrane particularly of the epithelial cells [8]. Furthermore, predecidual cells may be seen around the arterioles. Accompanying the predecidual alternation there is stromal regression and loss of endometrial height. In the absence of pregnancy, gland secretion reduces and involution of the epithelial gland occurs.

1.2.1.3 Menstrual stage

The menstrual cycle commences from the first day of the menstrual bleeding, and the upper two thirds of the endometrium (functionalis layer) are shed. It

appears that the menstrual tissue is the result of the enzymatic autodigestion and prostaglandin related ischemic necrosis of non-gestational oestrogen/progesterone primed endometrium[9]. The remaining endometrium (deep functionalis and basalis layer) begin the regeneration from the third day and starts in the glandular and stromal elements [10] (Figure 1.1).

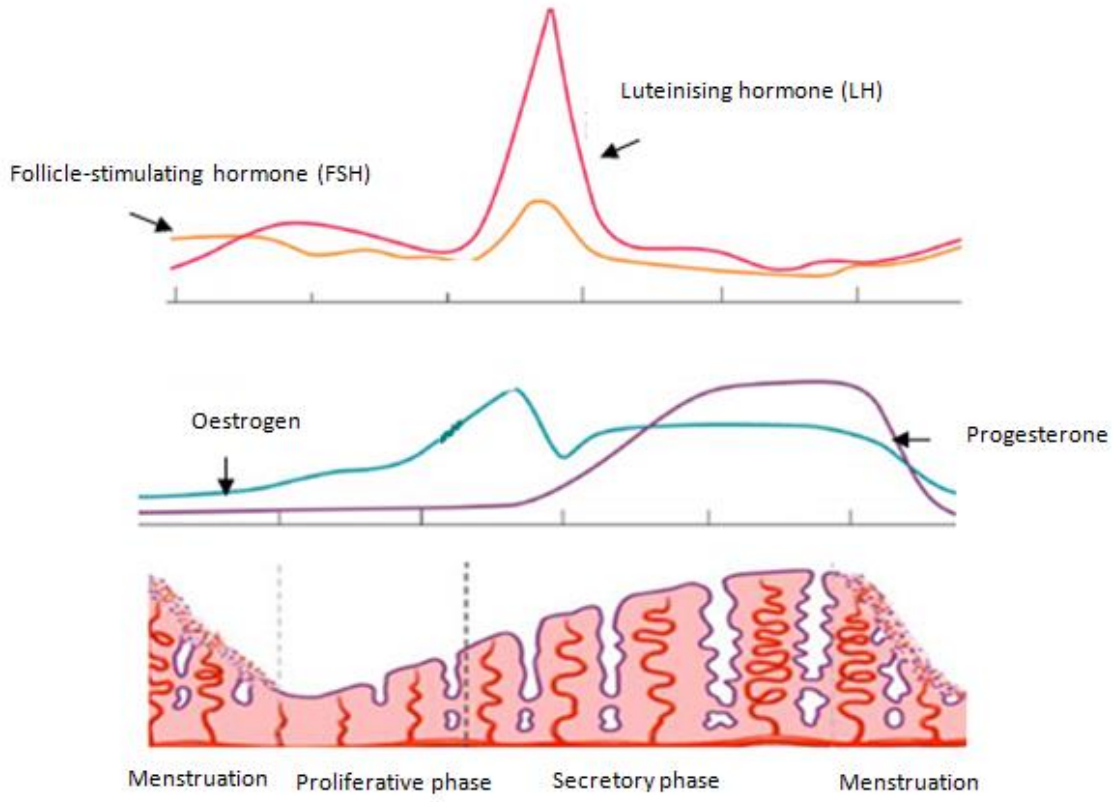


Figure 1.1: Depiction of changes at the endometrial level for hormonal fluctuations throughout the menstrual cycle.

1.2.2 **Endometrial blood vessels**

The endometrium is supplied by the radial branches of the arcuate arteries in the myometrium which derive from the uterine arteries. This blood supply is critical for menstruation. The blood vessels in the upper two third of the functional layer of endometrium are characterised by their spiral shape which is unique for menstruating species [11]. Such vasculatures are involved in the mechanism of menstruation by being involved in leukocytes influx and vasoconstriction. Furthermore, these blood vessels undergo cyclical changes such as dramatic growth, disruption, re-modelling and repair [12]. These vascular changes are a part of preparing the endometrium for the implantation

process, and supporting a healthy pregnancy, but if pregnancy does not occur, the vascular changes are associated with per-vaginal bleeding.

These arterioles have a strong structure due to a tight connection between the endothelial cells and basement membrane. They are highly sensitive to ovarian hormones and, therefore, thought to be controlled with the steroid hormones [13]. The growth and the differentiation of these blood vessels are mediated in the presence of oestrogen receptors (ER) and progesterone receptors (PR) within the smooth muscle cells. However, other observations have demonstrated that endothelial cells in the endometrium do not express ERα and PR but do express ERß [14]. Therefore the effect of steroid hormones on endometrial blood vessels may occur in an indirect way mediated by local molecules and certain components of the basement membrane such as heparin sulphate. This reduces the stabilisation of the blood vessels and it shows decrease during the menstrual phase of the cycle [15]. Also, fibronectin[16] and thrombospondin[17] , in addition to various matrix metalloproteinases (MMPs),which can breakdown the basement membrane are involved.

There are leukocyte populations present within the endometrial stroma. These populations vary in type and number in the endometrium across the menstrual cycle and throughout the pregnancy. Endometrial leukocytes include T and B cells, mast cells, neutrophils, macrophages, and uterine natural killer cells (uNk). Details about each immune cell are presented later in this thesis(chapter 4). Regardless of the variation in endometrial leukocyte populations within the endometrium throughout the menstrual cycle, interestingly, all of them increase significantly in late secretory phase and during the menstruation. These cyclical changes in immune cell number implicate direct or indirect regulation by sex steroid hormones, whether oestrogen and /or progesterone. However, the majority of leukocyte populations do not express the classic ERα or PR. Therefore, the control of their appearance by the ovarian sex hormones is most likely to be indirect. Nevertheless, uNK immune cells display ERß and glucocorticoid receptors (GR) [18],which raisesthe possibility concept that oestrogen and glucocorticoids act directly on uNK cells to influence gene transcription in the endometrium and decidua [18].

Monoclonal antibodies used in immunohistochemicalanalysishave a vital role in distinguishing endometrial leukocyte from peripheral immune cells. However, it is not clear whether the increased immune cell number within the endometrial stroma is solely the result of in situ proliferation or whether there is also influx of immune cells from the peripheral circulation into the endometrium. In addition, many of the endometrial leukocytes display different phenotypes, and subtypes, where some have been described to express MMPs and to release cytokines and protease.

Each Endometrial leukocyte population has different roles in the endometrial stroma according to their time of up-regulation across the menstrual cycle, their ability to release or activate cytokines and their activation pathway. For instance, the largest leukocyte population in the endometrium during the late secretory phase and pregnancy comprises uNK cells [18, 19]. These cells are located in the endometrium in close contact with endometrial glands and spiral blood vessels, which suggest a role in vascular growth and remodelling [20]. In addition, the uNK cell is the main type of leukocyte in the endometrial stroma during implantation, placentation and decidualization[19, 21, 22]. These immune cells may have a strong relationship with the uterine stromal cells, as interleukin 15(IL15), prolactin and some soluble factors, which are released by the uterine stromal cells, may mediate the effect of oestrogen and progesterone on uNk cells [23, 24]. This relationship is supported by the strong association between ectopic decidua and presence of uNK cells [19].

Neutrophils generally have faint endometrial expression across the menstrual cycle, however, neutrophils show abundant expression immediately before menstruation[25]. This sudden influx of neutrophils into the endometrium is timed with the withdrawal of progesterone, and this raises a thought that withdrawal of progesterone triggers the infiltration of neutrophils into the endometrial stroma. Neutrophils have the ability to synthesise and release a wide range of immunoregulatory cytokines and hence initiate and augment the cellular and humoral immune response [26]. In addition, many studies have pointed out the role of neutrophils in mucosal defence as well as in menstruation [27, 28].

Another type of leukocyte in endometrium is the macrophage which displays a cyclical pattern of expression across the menstrual cycle, and corresponds to approximately 20% of endometrial leukocytes in the late secretory phase [22, 25]. Macrophages also produce a wide range of regulatory molecules which may have a role in stimulating production of pro-inflammatory cytokines from adjacent cells[25].

The Mast cell is another uterine leukocyte, although there is no change in the number and the distributions of mast cells across the menstrual cycle. Mast cells are activated in late secretory phase before menstruation, giving a suggestion of a role of mast cells in upregulation of MMP before the onset of menstruation [29-31]. Mast cells are characterised by its content of tryptase alone or tryptase and chymase[32].

1.3 Steroid hormones and their receptors in endometrium

The ovarian sex steroid hormones, oestrogen and progesterone have the main role in the cyclical endometrial proliferation and degeneration. These endometrial changes are under the control of variation in steroid hormones concentration[33]. The response to this fluctuation in steroid hormones is mediated by oestrogen and progesterone receptors (ER and PR respectively). The classical oestrogen and progesterone receptors are nuclear receptors ,which localise between the cytoplasm and the nucleus[34].

The endometrium contains stromal, epithelial glandular and endometrial vascular cells, in addition to perivascular endometrial cells. The sex steroid receptors have been expressed temporally and spatially in the endometrium [14]. Endometrial proliferation is under the control of oestrogen and endometrial repair, therefore, is thought to be an oestrogenic event. Progesterone, on the other hand, exerts anti-oestrogenic effects by inhibiting the endometrial proliferation and enhancing the glandular differentiation. However, interestingly, these progesterone effects are mediated through its receptors, which only exist in oestrogen primed endometrium. Thus, the presence of PR indicates a functional ER pathway. Both oestrogen and progesterone receptors are up-

regulated in stromal and epithelial glandular cells during the proliferative phase, while ER reaches the peak of its expression in the late proliferative/early secretory phase. During the secretory phase, there is reduction in the expression of both oestrogen and progesterone receptors particularly in epithelial glands due to the action of progesterone at the transcriptional and post-transcriptional level.

Oestrogen and progesterone receptors are expressed in different subtypes. For instance, the ER has two subtypes known as ERα and ERβ, each of them resulted from distinct genes. Furthermore, their level of expression is variable in different cellular compartment in the endometrium. ERβ exist in splice variants ERβ1 and ERβ$_{CX}$/ ERβ2 in the endometrial stromal cells and the epithelial glands in both functional and basal layers of endometrium. In the smooth muscle of the uterine wall, the expression of ERα and ERβ is demonstrated during the proliferative phase with higher expression of ERα than the expression of ERβ. On the other hand, the ERα mRNA and protein are reduced in its expression within the functional layer of the endometrium during the secretory phase, and characterised by sharp decline in the stromal cells, although there is a gradual decline in epithelial glandular cells[35]. ERβ1 maintains its level across the menstrual cycle, and ERβ$_{CX}$/β2 decreases in the glandular epithelial of the functional layer in the mid-secretory phase [36, 37]. ERα has been identified in perivascular cells but not in the endothelial cells. On the other hand, ERβ is largely detected in endothelial cells of blood vessels within the endometrium in late secretory phase and in the perivascular cells as well as [14, 38]. In addition, ERβ has been found in the uNK cells in endometrium [18].

The progesterone receptor is also present in two subtypes, known as PRA and PRB. In contrast to the ER, PR subtypes are transcribed from two different promoters that originated from one single gene. The PRA has a shorter amino acids chain than that of PRβ. By there, the PRA is considered to be a shorter form of PRB. Each isoform has an individual transactivational function which is different for each cell type and ligand. However, PRB is transcriptionally more active than PRA [39, 40]. On the other hand, PRA can block PRβ and stop the ER activity in several instances. Both isoform are up-regulated during the mid to late proliferative phase in both endometrial glandular and stromal cells. In addition, both of them are decline in endometrial glandular cells during the

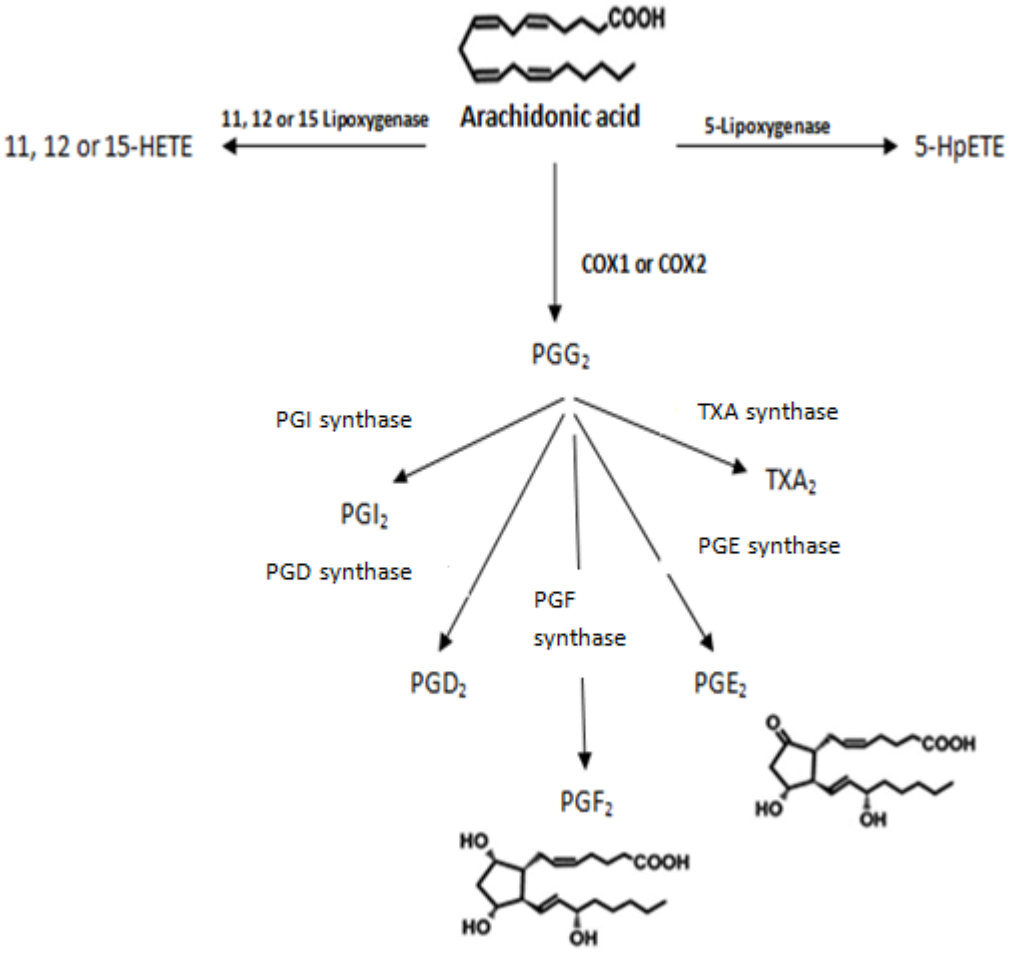
secretory phase due to high levels of progesterone. Nevertheless, PRA remains prominent in the stromal compartment mainly around the endometrial vasculature during the secretory phase, and could be the main isoform mediating the progesterone action in endometrium during this phase [41, 42]. Both PR subtypes are not present in endometrial endothelial cells or leukocytes, indicating an indirect action of progesterone on them. PR receptors are expressed in the basalis layer of endometrium throughout the menstrual cycle, demonstrating different regulatory factors of this deeper layer of the endometrium than these of the superficial shedding layer [35].

Whereas the transcriptional effect of oestrogen and progesterone is mediated by classical nuclear receptors, alternative membrane receptors may be involved in their non-genomic action [43, 44]. Furthermore, in previous study, fast signal beyond the nuclear compartment was noted for both oestrogen and progesterone, which may be mediated via non-nuclear transduction pathway [45].

1.4 Prostaglandins

Prostaglandins are lipid-derived eicosanoids that modulate many biological functions in several body systems including the CNS, cardiovascular, gastrointestinal, genitourinary, endocrine, respiratory, reproductive, and immune systems[46]. In addition, the prostaglandins have been implicated in a wide range of diseases including cancer, inflammation, cardiovascular disease, hypertension, and reproductive dysfunctions. Prostaglandins are potent bioactive messengers derived from the arachidonic acid (AA), which first extracted from the male reproductive tract in 1930s, and noted to decrease the blood pressure and cause smooth muscle contraction. The first prostaglandin isomers were identified in 1950s and 1960s, and may be divided according to the substituents in their cyclopentane ring into the A, B, C, D, E, F or J series. Later arachidonic acid (20-carbon tetraenoic fatty acid) was identified as precursorof prostaglandins, and the later known as a C_{20} carboxylic acid related family. The activation of phospholipase enzyme results in liberation of the arachidonic acid from plasma membrane phospholipids or dietary fat. The cyclooxygenase (COX) reaction through which AA is enzymatically cyclised and oxygenated to yield

endoperoxide-containing prostaglandin G2 (PGG2) is completed by adding the 15-hydro-peroxy group, and then followed by a reduction in that group to a hydroxyl group via a separate peroxidase active site on the enzyme, as result an intermediate form of prostaglandins known as prostaglandin H_2 (PGH_2) is formed [47, 48] (Figure 1.5). Isomerases and oxidoreductases produce various bioactive prostaglandin isomers using PGH2 as substrate, and these are named according to the synthase enzyme, for instance, prostaglandin E_2(PGE_2) is synthesised by prostaglandin-E-synthase (PGES), prostaglandin F2α (PGF2α) is synthesised by prostaglandin-F-synthase (PGFS),prostaglandin D (PGD) is produced by prostaglandin-D-synthase (PGDS),prostacyclin (PGI_2) by prostaglandin-I-synthase (PGIS),and thromboxane A2 (TXA2) by thromboxane synthase (TXS)[46, 49].



Figur1.2: Depiction of Prostaglandins synthesis from arachidonic acid by cyclooxygenase pathway (cyclooxygenase1 and 2, Cox1 & COX2 respectively). Prostaglandin G2 as precursor to other types of prostaglandins as following: prostaglandinE_2 (PGE_2) by prostaglandin-E-synthase (PGES), prostaglandin F2α (PGF2α) by prostaglandin-F-synthase (PGFS), prostaglandin D (PGD) by prostaglandin-D-synthase (PGDS), prostacyclin (PGI_2) by prostaglandin-I-synthase (PGIS), and thromboxane A2 (TXA2) by thromboxane synthase (TXS). 5-hydroperoxyeicosatetraenoic acid (5- HPETE) synthesis by 5-lipoxygenase and 11, 12&15Hydroxyeicosatetraenoic acid (11, 12&15-HETE) synthesis by11, 12 or 15-lipoxygenase.

Induction of prostaglandin by mitogens and proinflammatory agents, as well as down-regulation of prostaglandin by glucocorticoids was the most presenting data regarding the potential of more than one COX behind this. In addition, observational data indicated that prostaglandin synthesis and release is different in some situations, such as in activated platelets, where it occurs within a few minutes after stimulation. In other cases, such as in mitogen-stimulated fibroblasts, prostaglandin synthesis may take longer (hours) to occur.

Furthermore, many investigators observed that prostaglandin induction as well as increased level of PG can be occurred without or with minimal an increase in the seminal vesicle COX enzyme which was found to be present in most cells and tissues investigated [50]. Also the action of glucocorticoids on PG synthesis and seminal vesicle COX levels is not completely understood, which strongly decreased PG synthesis but generally had little to no effect on seminal vesicle COX levels. Various hypothesises were suggested a consistencybetween these observed phenomena, and the most common of which was that changes in substrate delivery were responsible for these fluctuations in PG synthesis. Some authors haveconcluded that monocytes may contain two pools of COX under lipopolysaccharide stimulation, witheach of them with a different sensitivity to the lipopolysaccharide. Another study used giant two-dimensional protein gel electrophoresis, proteins immunoreactive with COX-1 antibodies that were induced in v-src-transformed cells[51, 52]. The evidence in these and other early studies was consistent with two different inducible and constitutive COX isozymes encoded by separate genes but was also compatible with other explanations. Studies of cell division helped to demonstrate the mechanism by which COX enzymes upregulate the PG production in inflammation and other physiological condition [53, 54]. Later on, a new inducible COX enzyme was reported from studies in chicken and mouse [55, 56], contributing to the identification of COX-2. Thus the inducible enzyme cloned in these studies referred to as COX-2 and the seminal vesicle form of the enzyme as COX-1. Different COX-1 splice variants have been identified in different species, and one of these is a brain specific splice variant identified in dog and termed COX3. This consists of an intact and unspliced COX-1 transcriptthat retains intron-1.The COX-3 variant produces protein thought to havethe ability to reduceprostaglandin synthesis relative to COX-1. Analgesic/antipyretic drugs such as acetaminophen and dipyrone favourably inhibit this activity[57].

The biological action of prostaglandins is mediated through their receptors, which are designated by the letter P and a prefix D, E, F, I and T, and have the form DP, EP, FP, IP and TP referring to PGD, PGE, PGF, PGI and TXA_2respectively. PGE2 exerts its effect through four subtype receptors, EP1, EP2, EP3, EP4. These receptors are G-protein coupled receptors using alternate and sometimes opposing intracellular pathways. These receptors are derived from a separate gene, with extra splice variant for EP3,FP, and TP which are different in their C-terminal [58]. FP, TP, and EP1 receptors specially couple in response to an increase in cell calcium and induce Ca mobilisation via $G_{\alpha q}$, leading to smooth

muscle contraction to be contractile receptors. EP2, EP4, DP, and IP receptors stimulate cyclic adenosine monophosphate (cAMP) via Gαs and are called relaxant receptors as they cause smooth muscle relaxation, whereas the EP3 receptor preferentially couples to Gi, inhibiting cyclic AMP generation, and is thus called an inhibitory receptor which causes smooth muscle contraction. However, some of EP3 splice variants in specific cell types can elevate the cAMP [58].

In addition to the prostanoids that act principally via plasma membrane-derived G-protein-coupled receptors, several COX products can activate nuclear receptors of specific classes, and stimulate the nuclear receptor pathway [59]. It was found that these nuclear-acting prostanoid ligands inhibit the IκB kinase activity and thereby block the NFκB transcription factor pathway [60], suggesting that nuclear-acting prostanoids may act to down-regulate angiogenesis [61],and raise the possibility that the COX pathway may induce anti-angiogenic effects by nuclear-acting prostanoids. Therefore,a number of COX-2-selective inhibitors have been developed to control the anti-inflammatory and anti-neoplastic activities of the COX-2 isoenzyme. Inhibition of the COX isoenzyme activity and/or expression may be the basis of future development of anti-inflammatory and anti-neoplastic drugs.

Cross communication between some prostanoid receptors (EP2 and FP) and epidermal growth factors receptors has been demonstrated, resulting in activation of receptor tyrosine kinases (RTKs). The signal mechanism behind this activation is not fully elucidated. However, many intracellular and extracellular mechanisms have been suggested involves auto-phosphorylation of RTKs, activation of transmembrane matrix metalloproteinase and extracellular release of heparin binding epidermal growth factor (HB-EGF),and phosphorylation of the mitogen activated protein kinase extracellular signal-regulated kinase 1 and 2 signalling pathway [62, 63].

The physiological role of prostanoids is complex.However, recent progress in understanding both the disruption of the effects of respective gene and the use of receptor selective compounds gives insight into the physiological role for each receptor. It has been demonstrated that each prostanoid receptor has multiple functions, and that their expression is regulated in a context dependent manner

that sometimes results in opposite, excitatory and inhibitory, outcomes. In addition, the balance of prostanoid production and receptor expression has been demonstrated to be important for homeostasis of the human body[64].

1.5 Vascular endothelial growth factor (VEGF)

This cytokine is produced by endothelial cells is hypoxic conditions and it exerts its effect by binding to two types of tyrosinase kinase receptors on the endothelial cells. These are VGEF receptor 1, which is responsible of the organization of endothelial cells in tubes, and VGEF receptor 2, that induces endothelial cell migration and proliferation. Both receptors are upregulated in a hypoxic environment (Figure 1.8). VGEF is a glycoprotein and has a role in enhancing the mitosis of endothelial cells as well as their permeability. The most important form is VEGF 165.

1.6 Cytokines (interleukin 8)

Interleukin (IL)-8 is a member of the C-X-C family of chemokines that has high affinity binding to G- protein coupled receptors, IL8-1 (CXCR1) and IL8-2(CXCR2), a G-protein-activated second messenger system. [65, 66]. The IL8-1 is more specificIL-8, while the CXCR2 is less specific to IL8 and responds to several additional chemokines including growth-related protein-α (GRPα), neutrophil-activating peptide-2, and epithelial-derived neutrophil attractant-78[65, 67]. The common characteristic shared by all chemokines that activate the CXCR2 is a specific sequence in the amino terminus, which seems to function as a recognition sequence for receptor binding and activation.

Early studies concentrated on the effect of IL-8 on neutrophils. It has been found that neutrophils respond to IL-8 in different mechanisms including, calcium mobilisation, actin polymerisation, enzyme release and chemotaxis [68, 69].It is thought that CXCR2 may play an active role in the initiation of neutrophil migration distant from the site of inflammation, where the concentration of IL-8 is at the picomolar level. The low affinity CXCR1 may play a more active role in mediating IL-8 signal at the site of inflammation, where the concentration of IL-

8 is high[70],indicating that neutrophil chemotaxis is primarily mediated by the CXCR1.

Apart from neutrophils, many cell types have been shown to express IL-8 receptors. These cell types include monocytes, macrophages, and endothelial cells [71, 72]. Although activation of the CXCR2 can enhance cell proliferation oftumour cells[73], the physiological role of IL-8 receptors on non-hematopoietic cells is not fully understood, however, a role in cell movement is expected [74].

IL8 like other related chemokines, which have NH2 ELR sequence, may have angiogenic properties. It has been suggested that the CXCR2 is the endothelial cell receptor that mediates this angiogenic response, because of the chemotaxicproperty of IL8; it causes cytoskeletal rearrangement due to activation of both the CXCR1 and the CXCR2. However, both temporal and qualitative differences exist between the behaviour of the two receptors. Consequently, progressive receptor activation through two signalling pathways may regulate endothelial cell cytoskeletal responses to IL-8. In addition, in fibroblast, through activation the small G protiens (Cdc42, Rac, and Rhc), IL8 may help in forming a meshwork of actin filament at the leading edge of migrating cells [75]. Furthermore, formation of stress fibre can be enhanced by the activation of Rho protein, and then inserted into focal adhesion complexes. In fibroblasts, the activities of these three proteins thought to be arranged in a ranked manner, where activation of one of them turns to activate the other. However, depending on cell type and possibly the activation pathway, the relationship between the different small G proteins may vary. In the endothelial cells, activation of Rac protein by thrombin leads to endothelial cells retraction [76]. In addition, it was reported that IL-8 initially activates Rho and actin stress fibre formation in endothelial cells due to activation of the CXCR1. Afterward, Rac is activated in a CXCR2-dependent manner, leading to cell retraction and gap formation between adjacent cells.

1.7 Angiogenesis in the normal endometrium

Angiogenesis is the development of new microvessels from existing vessels and it involves microvascular endothelial cells. Physiological angiogenesis rarely occurs

in adults except during episodes of wound healing, and in the ovary and the endometrium during the reproductive life of females. Angiogenesis is vital for development and differentiation of human endometrium that are necessary for implantation, as well as for maintenance of pregnancy [77].The rapid cyclic growth of the endometrium demands a dynamic changing of its vascular supply. A complex process of vascular and glandular proliferation, differentiation, sloughing and regeneration occurs each month. These periodic alterations are applied to most of endometrial compartments and are controlled largely by ovarian steroids. Theses massive changing throughout the menstrual cycle (discussed above in the proliferative and secretory phases), including increased glandular cells, stromal fibroblasts, and vascular endothelial cells, and enlargement of endometrial glands during the proliferative phase and differentiation to cells of the functional layer of the endometrium, increased the ratio of glands to stroma, stromal oedema and stromal cell decidualization take place during the secretory phase. This cyclic growth of the endometrium is accompanied by extensive vascular modification in the horizontal branches supplying the basal layer and vertical branches supplying the functional layer, and more obvious in the spiral arteries and end-arterioles, which play a critical role in the regulation of uterine bleeding during the menstrual cycle. These end arterioles are highly sensitive to ovarian steroids, where it is noticed that the endometrial vascular architecture changes throughout the menstrual cycle, corresponding to the changes in the uterine epithelium and stroma. Moreover, there is a gradual increase in branching and coiling of spiral arterioles, in parallel to an increase in the length and coiling of endometrial glands. Vasoconstriction of the distal segments of the spiral arteries occurs in response to progesterone withdrawal, and 4 days laterdiffuse necrosis, inflammation, and vascular thrombosis occurs resulting in menstruation.

The serial events that lead to angiogenesis begin through stimulation of the endothelial cell by hypoxia. As consequence, activation of the endothelial cell with production of vascular growing factors takes place.Migration of the endothelial cells with disruption of the basement membrane seems to be a first event, followed by proliferation and the formation of tubes with periendothelial support. Subsequently a new blood vessel is formed. The promotion of migration of endothelial cells is a complex process where proteases like plasmin have a critical role in stimulating the migration of endothelial cells across the matrix of

the cells. Multiple studies have demonstrated the importance of smooth muscle cells together with endothelial cells in the process of developing new blood vessels (Figure1.3). It is thought that proliferation of smooth muscle cells is an intrinsic element in angiogenesis. Many of these mechanisms are mediated through cytokines, which act by binding to tyrosinase kinase membrane receptors.

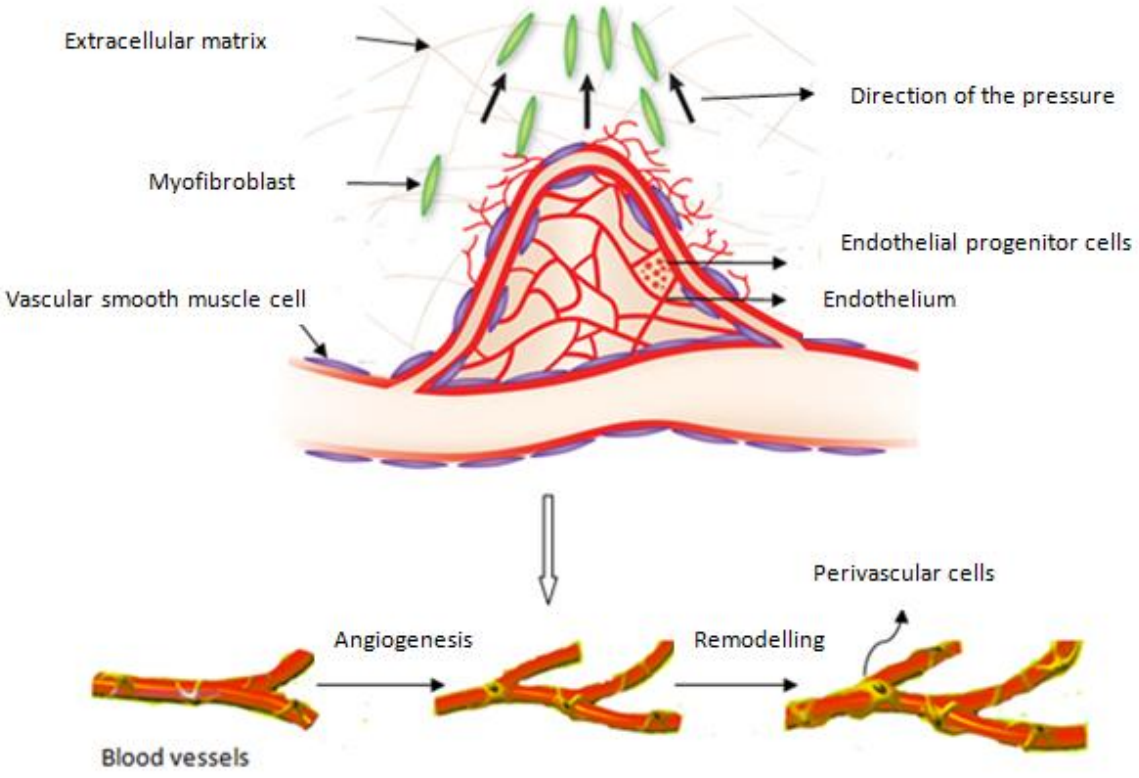


Figure 1.3: Figure showing the migration and proliferation of endothelial cells and smooth muscle cells during the formation of new blood vessels from existing blood vessels.

1.7.1 **Growth factors and angiogenesis**

Following menstruation, endometrial vessels in the shedding layer need to be reconstructed. Therefore, development of endothelial cells and smooth muscle cells is promoted to form capillaries and larger vessels [10]. This process of new vasculaisation is promoted by several angiogenic factors [78]. The main angiogenic factor is vascular endothelial growth factor (VEGF), also termed vascular angiogenic factor.

It is known that ovarian steroid hormones regulate changes in the human endometrium during the menstrual cycle, and endometrial cells are very sensitive to oestrogen. Growth factors can regulate endometrial proliferation

and differentiation and may mediate the effects of oestrogen and progesterone on the endometrium in an autocrine and /or paracrine manner. Although the effects of growth factors and sex steroids on epithelial and stromal development have been investigated in several studies, little is known about factors that affect the endometrial vasculature throughout the menstrual cycle. It is thought that endothelial cells do not respond directly to oestrogen or progesterone, and growth factors may play a role as local mediators of the effects of ovarian steroids on endometrial vessels. The angiogenic basic fibroblast growth factor (bFGF) has been excluded as mediator of endometrial angiogenesis, as its levels do not change in human endometrial biopsies during the menstrual cycle and increase after menopause. On the other hand, vascular endothelial growth factor (VEGF) peptide and mRNA are present in human endometrial biopsies obtained during all phases of the menstrual cycle and VEGF is localised to both glandular epithelium and stroma. Consistent with the hypothesis that VEGF may be a paracrine regulator of the effects of sex steroids on endometrial angiogenesis, VEGF gene expression is increased by the addition of estradiol to human endometrial carcinoma cell lines. Estradiol also increases VEGF expression by primary human endometrial cells in culture. These observations suggest that VEGF may play an important role in the regulation of endometrial angiogenesis throughout the human reproductive cycle (Figure 1.4). Therefore, VEGF is thought to play a crucial role as endothelial cells growth and survival promoter. It appears that VEGF expression in the whole endometrium is low during the proliferative phase, increases during the secretory phase and reaches the maximum levels during menstruation. This supports a hypothesis which suggests induction of hypoxia rising from constriction of spiral arterioles, which precedes bleeding, leads to increased production of VEGF by endothelial cells to promote angiogenesis. However, there is no clear pattern of VEGF protein expression throughout the menstrual cycle, although expression is greater in glands than stroma.

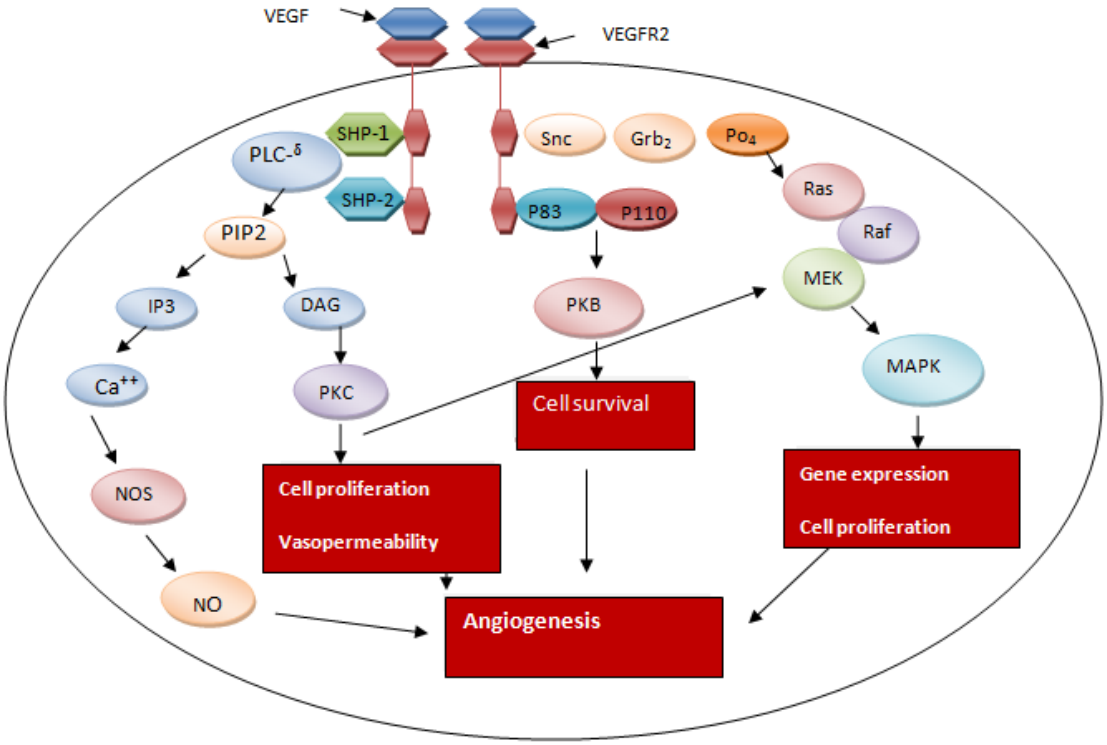


Figure1.4: Figure showing the VEGF signalling pathway in endothelial cell

1.7.2 **Cyclooxygenases and angiogenesis**

Cyclooxygenase enzymes (COX) (prostaglandin H2 synthase) are the enzymes responsible for the conversion of arachidonic acid from membrane phospholipids to prostaglandin H2 (PGH2), the common precursor of various forms of PGs. The main two COX isomers, COX-1 and COX-2 consist of 600-602 (depending on the species) and 604 amino acids, respectively. Despite a similarity in the primary structures, the isoenzymes show differences in the patterns of expression and cellular function. COX-1 is found constitutively expressed in most mammalian tissues and helps in maintenance of the cellular physiological functions, such as platelet aggregation and cell homeostasis. COX-2 is normally expressed at a very low level in most tissues and is highly inducible by growth factors, cytokines, and tumour promoters. Cyclooxygenase-2 exerts its effects through its reaction products. Cyclooxygenase-2 is found to be expressed in newly formed blood vessels within tumours grown in animals, which suggested that cyclooxygenase-2

modulates angiogenesis by augmenting the release of angiogenic peptides, such as VEGF, thymidine phosphorylase, bFGF, and nitric oxide. On the other hand, VEGF can induce COX-2 via multiple mechanisms and that COX-2 can increase VEGF via hypoxia inducible factor-1 (HIF-1). The role of COX-2 enzyme in angiogenesis further exacerbated by decreasing the anti-angiogenic factor

1.7.3 **Prostaglandins and angiogenesis**

Prostaglandins are part of a family of biologically active lipids derived from arachidonic acid by the action of phospholipase. Terminal PGs are subsequently produced by specific prostaglandin synthase enzymes from PGH2. In the case of PGE2 and PGF2α, it was found that microsomal prostaglandin E synthase-1 and -2, cytosolic prostaglandin E synthase, and prostaglandin F synthase are expressed in the human endometrium. Prostaglandins have a primary role in pathological conditions such as inflammation, hypertension and cancer but are also essential for normal physiological function such as in the female reproductive system. Prostaglandins are important regulators of endometrial function. Their secretion is controlled by both endocrine and paracrine mediators. Potential paracrine regulators of endometrial origin include cytokines, growth factors and histamine. Therefore, the levels of prostaglandins in the human endometrium throughout the normal menstrual cycle are variable. For instance, the levels of prostaglandin F2α and prostaglandin E2 are low during the proliferative phase of the cycle and both of them have nearly similar level in this phase. However, prostaglandin E2 rises significantly during the secretory phase, put remains lower than the level of F2α during the secretory phase. The prostaglandin E2 level is highest at menstruation. Dysregulation of endometrial prostaglandins production can cause menstrual cycle disorders, infertility and uterine malignancies. PGs are not stored within cells, but are synthesized as required in response to stimuli. The first step in their synthesis is the release of arachidonic acid from the cellular phospholipids, by the action of the enzyme phospholipase A2. Following biosynthesis, prostaglandins exert their function through G protein coupled receptors. If COX-2 activity increases in cells, its metabolite (PGE2), affects metabolic pathways via specific receptor subtypes (EP1-EP4 for PGE2), EP2 is likely important in the epithelial cell compartment, whereas EP4 is highly

expressed in stromal and hematopoietic cells. Both receptors are coupled to the heterotrimeric Gs protein and regulate the cAMP/protein kinase-A pathway. It is thought that cyclooxygenase 2 (COX-2) COX-2 in the epithelial compartment signals via the EP2 receptor in an autocrine manner and via the EP4 receptor in a paracrine manner. PGE2 is a potent inducer of angiogensis and induces the expression of angiogenic regulatory proteins such as VEGF. On the other hand, it sis suggested that VEGF induce COX-2 mRNA expression, hence it increases the PGE2 concentration in the surrounding area. Afterward, PGE2 enhance the expression of VEGF. So it seems that PGE2 directly stimulates the angiogenesis, a part of VEGF signalling, and further induces VEGF expression. In addition, the effect of VEGF on angiogensis may be mediated by PGE2 secretion. . It is thought that PGE2 may up-regulate HIF-1 mRNA and protein via the EP2 receptor and this upregulation is dependent upon epidermal growth factor receptor (EGFR) kinase activity. The endometrial HIF-1 activation may occur via a PGE2-regulated pathway.

1.8 The role endometrial immune cells in angiogenesis

It is well known that the hormonal and local immune response is involved in the recognition and maintenance of pregnancy and it is suggested that alternations in the local immune system are controlled by steroid hormones, secreted by the ovary and placenta. When no pregnancy happens, the same immune cells are involved in changes in the endometrium that result in menstruation.
The withdrawal of progesterone from the endometrium is associated with features similar to an inflammatory event, where this up-regulate some inflammatory mediators and induce COX-2. These local events result in an elevation of local prostaglandin concentrations (PGE and PGF2α) and there may be synergism with the chemokine IL-8. The substantial peri-menstrual influx of leucocytes consists of neutrophils, macrophages and other immune cells that influx from circulation. These leukocytes themselves are sources of cytokines that further augment leukocyte influx. There is infiltration of leukocytes and release of prostaglandins as well as development of stromal oedema. Interleukin 8(IL-8) as a-chemokine, is chemotactic for both neutrophils and natural killer cells (NK), having a perivascular setting in the human endometrium.

Furthermore, IL-8 mRNA expression is also localised to the perivascular cells of late secretory endometrium. IL-8 is produced by monocytes and fibroblasts and there is evidence that it has an angiogenic character. Furthermore, the action of IL-8 may directly affect endometrial cell proliferation. The important relationship between PGE2 and IL8 may have an important effect on neutrophil chemotaxis, which is markedly elevated by low levels of PGE.
Most studies have not demonstrated the connection between expressions of endometrial angiogenic factors and new vessel growth. However, it was found recently that a strong relationship exists between vascular endothelial growth factor (VEGF) immunolocalised in intravascular neutrophils and endothelial cell proliferation in each of the subepithelial capillary plexus, functionalis and basalis regions of the human endometrium. There is an indication that the neutrophil VEGF has a role in the development of the subepithelial capillary plexus and functionalis microvessels during the proliferative phase of the menstrual cycle. It has been suggested that neutrophils are an intravascular source of VEGF for vessels that undergo angiogenesis by intussusception and elongation.

1.9 Menstruation:

Human endometrium is subjective to cyclical changes throughout a women's reproductive life. These changes represented in injury and repair and are regulated by steroid hormones. Oestrogen and progesterone are the two principal sex steroids influencing endometrial growth, differentiation and function, and they are implicated in endometrium pathology as well. Nowadays, there is a great knowledge about the role of steroid hormones in the onset of menstruation and in the process of endometrial repair. Simply, menstruation is a shedding of the superficial layer (functional layer) of the endometrium. Sequential events occur before, during and after this vital event. Menstruation is a complicated process that has been described as inflammatory pathway [79].

1.9.1 The mechanism of normal menstruation

The menstrual cycle is a physiological process regulated by a complex hormonal system with positive and negative feedback mechanisms. This mechanism is

associated with changes in sensitivity of peripheral tissues. Regular, pulsatile secretion of gonadotrophin releasing hormone (GnRH) from the hypothalamus is the primary event to drive the system. In response to this secretion, the pituitary gland releases follicle stimulating hormone (FSH) and luteinizing hormone (LH). The FSH facilitates the action of LH on the ovary, which in turn permits the synthesis of estradiol and progesterone, following by negative feedback of estradiol and inhibin on the hypophysis cerebri. Ovulation is caused by peak levels of LH, but it is dependent on progesterone levels. Synthesis of progesterone by the corpus luteum precedes the negative feedback of progesterone at the hypothalamic level. At the endometrial level (the functional layer) the number of sex steroid receptors increases during the proliferative phase and decreases during the secretory phase. In the absence of pregnancy, the corpus luteum is collapsed leading to a rapid decline in the ovarian steroids hormones, which triggers a compound of events within the functionalis layer of endometrium. Induction of menstruation was blocked by adding-back progesterone up to 36 hours following the decline in steroid hormones, but no suppression of menstruation occurred after 36 hours [80]. This observation gives a suggestion that menstruation occurs as result of withdrawal of progesterone, and the different up-regulation of PR across the menstrual cycle may influence this action as an early events through PR- positive cells and later it depends on PR –negative cells.

Markee and his colleagues in 1978 transplanted a fragment of human endometrium into the anterior chamber (iris) of rhesus monkey’s eyes and his direct observations have provided the initial understanding of the mechanism of menstruation. The development of the implanted graft involved progressive episodes including, increased the coiling of spiral arterioles, vascular stasis, alternation of vasoconstriction and vasodilatation episodes, and perivascular bleeding. Marked regression of the developed grafts was noted after oestrogen and progesterone withdrawal [11]. All these previous events may be caused and controlled by wide range of molecular and cellular mechanisms. Some of these mechanisms are well understood and others need to be fully elucidated.

Proinflmmatory mediators, such as prostaglandins and cytokines, for instance, are produced by perivascular cells in response to the concentration of progesterone. In addition, the spiral arterioles themselves may have a role in menstruation through a vasoconstriction and enhance leukocytes influx into the endometrium. The prostaglandins act as proinflammatory mediators and they are locally produced from the arachidonic acid by the action of cyclooxygenase 1 and 2 .The expression of prostaglandins in many compartments of endometrium varies across the menstrual cycle and tends to increase during menstruation. For instance, prostaglandin $F_2\alpha$ ($PGF_2\alpha$) causes vasoconstriction of blood vessels and leads to reduction in the blood flow to the endometrium[33]. $PGF_2\alpha$ is upregulated during the mid-secretory phase and still has high expression during the menstruation. On the other hand, prostaglandin E_2 is responsible for vasodilatation and it is mainly increased during the menstruation with less concentration than that of $PGF_2\alpha$ [81]. Upregulation of prostaglandins is associated with increase in the expression of locally acting mediators such as interleukins, angiogenic factors, and protease enzymes all these mediators which thought to have a role in infiltration of leukocytes into the endometrium and some leukocytes can release and activate cytokines as well.
All these events have guided authors who searching the menstrual event to describe the mechanism of menstruation process as interaction between ovarian hormones and immune system [82] and in other ward it is an inflammatory process under the control of the ovarian hormones[79].

1.10 Heavy menstrual bleeding (HMB)

Menstruation has been described as an inflammatory event with a complex interaction of sex steroids, prostanoids and cytokines leading to tissue degradation followed by a coordinated process of tissue regeneration and repair. The mechanism starts with progesterone withdrawal of which leads to up-regulation of COX-2 and subsequent generation of the potent vasoactive prostaglandins PGE2 (a vasodilator) and PGF2α (a vasoconstrictor). Heavy menstrual bleeding, which was formally known as menorrhagia, is defined as excessive menstrual blood loss that interferes with women’s physical, emotional, social and quality of life[83].

In 1966 a Swedish study, which was based on measuring the haemoglobin in the menstrual blood loss, found that the average of menstrual blood loss was about 40 ml, and that the incidence of anaemia among women who complained of heavy menstrual bleeding increased with menstrual blood loss more than 60ml per menstrual cycle and 90% centile of the distribution represented the cut-off point in the objective defining of heavy menstrual bleeding (≥80ml/per menstrual cycle)[84]. Nevertheless, clinically, approximately 50% of women complaining of HMB have objectively measured blood loss of 80ml or less[85]. A wide range of population may suffer HMB in their reproductive age (10-30%), which may broaden and affect half of them of perimenopausal age [86]. In the UK, nearly 800,000 women every year are seeking medical advice because of the highly socio-economic impact of this condition[83], and in earlier report about 5% of women would ask for medical advice because of the condition and fifth of those women are at risk of hysterectomy because of the condition[87].

1.10.1 Heavy menstrual bleeding in normal uteri

In the absence of microscopic and/or macroscopic appearance of uterine pathology, heavy menstrual bleeding known as idiopathic HMB, and clinically called dysfunction uterine bleeding [88]. The vast majority of women with HMB have normal endometrium. Nevertheless, a small proportion of those women may have a defective clotting mechanism, such as Von Willebrand's disease [89, 90] and deficiency of PAI [91]. Progesterone may have the ability to stimulate the protease inhibitors as plasminogen activator inhibitor -1 (PAI-1)[92]which can result in enhancing the thrombus formation. In normal menstruation, progesterone withdrawal, which is the key event in menstruation, may stimulate the secretion of plasminogen activator and opposite the previous action. Therefore, the reduction in the clotting function is a feature of menstruation, and clotting deficiencies diseases are most properly co-factors or a combination of factors resulting in HMB.

The incidence of HMB in women with normal uteri could be a result of disturbances in the local endometrial environment. This modification could be mediated by alternations in the expression, signalling and receptors of local

endometrial factors, which have important role in the establishment and maintenance of vascular haemostasis. Genetic factors may have a role in the heavy menstrual blood loss as well, where there was a link between the menstrual blood loss in monozygotic but not in dizygotic [93].

1.10.1.1 Cytokine alternations

It is widely agreed that menstruation is mainly an inflammatory response of endometrium to withdrawal of progesterone. Hence, an aberration in this mechanism can lead to heavy menstrual blood loss. The pro-inflammatory cytokine transforming growth factor-α (TGF-α) was found to be increased in menstrual effluent in women with heavy menstrual blood loss compared with that of women with normal menstrual blood loss[94].

Prostaglandins appear also to play important roles in the human endometrium, and in women diagnosed with heavy menstrual bleeding. The first demonstration of these markers was in menstrual fluid where vasoactive substances were demonstrated to posses the ability to induce contraction in strips of ileal muscle. Afterwards, these substances were identified and termed PGE_2 and $PGF_2\alpha$. These are the most abundant prostaglandins in endometrium and menstrual fluid [95].Their expressionis increased in the total PGs in endometrium of women with HMB [96]. Endometrial PGE_2 shows increase in its expression, signalling and the synthesis pathway in women with HMB. The endometrial expression of COX-2 during the secretory phase is also higher in women with HMB than in women with normal menstrual blood loss[97]. In addition, the number of PGE receptors (EP2and EP4) is greater in endometrium in women with HMB compared with that in the normal controls. PGE_2 synthesis and signalling have a direct correlation with the menstrual blood loss [98, 99]. Other vasodilatory factors such as prostacyclin (PGI_2) and nitric oxide synthesis are increased in endometrium in women with HMB and may up-regulate the PGE_2 synthesis pathway via a positive feedback mechanism [100].

On the other hand, prostaglandins up-regulate the expression of COX-2, arachidonic acid metabolism and prostanoids biosynthesis in many functional conditions. Because of the increased expression of prostanoids in endometrium of women with HMB, COX enzyme inhibitors have been introduced as mode of treatment. COX enzyme inhibitors may reduce the menstrual blood loss by 30%

[101-103]. Prevention of PG synthesis is the mechanism of action of non-steroidal anti-inflammatory drugs (NSAID), while the femanate group such as sodium meclofenamate and mefenamic acid works by reducing PG synthesis and suppressing PGE_2 binding to its receptors[104].

1.10.1.2 Angiogenic alternations

Local hypoxia,which follows withdrawal of progesterone has a crucial role in postmenstrual endometrial repair. The hypoxic inducible factor-1 (HIF-1) seems to co-ordinate tissue responses. This factor mediates its action through two subunits, HIF-1 α and HIF-1ß. Activation of HIF-1 which take place in absence of oxygen, enhances transcriptional activity of genes with hypoxic response elements, in particular factors which mediate endometrial remodelling and angiogenesis. The expression of HIF-1α has been observed in the endometrium during the late secretory and menstruation phases. Transcriptional activity, translocation, and protein stabilisation of HIF-1α,is thought to be enhanced by some inflammatory mediators such as tumour necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß), and PGE_2. The cytokine IL-1 has the ability to enhance the expression of hydroxyl steriod dehydrogenase-1 (11ßHSD-1)[105]. This enzyme converts cortisone to cortisol, which is an inflammatory steroid product and which has been observed expressed at high level during menstruation, suggesting its role in the postmenstruation repair. The action of this enzyme is reversed by type 2 enzyme (11BHSD-2), leading to decrease in the cortisol. The level of 11ßHDSD-2 is largely elevated in women with HMB [106], and consequently, the action of cortisol as an anti-inflammatory and angiostatic factor would decrease, which may result in dysfunction of angiogenesis in the endometrium and delayed repair. Therefore, menstruation as inflammatory process may have a direct impact on post-menstrual tissue repair.

Increases in vasodilatation and lack of vasoconstriction associated with HMB condition, may limit or prevent the perimenstrual hypoxic event in the regrowing zone of endometrium. As a result, the endometrial repair process may be delayed. Non hypoxic conditions cause inactivation of HIF-1, which may cause a decrease in the transcription of angiogenic repair factor, and subsequently may prolong the menstrual bleeding. VEGF is one of the genes targeted by HIF-1. Some authors found a decrease in the VEGF mRNA levels in the menstrual

effluent and in endometrium biopsied in women with HMB [94]. This low level of VEGF-A would detrimentally affect migration and differentiation of vascular smooth muscles and contributes to defective initiation of coagulation. As a result, delay in repair the damaged vessels and an increase in menstrual bleeding may result.

Upregulation of the expression of COX enzymes and prostaglandin receptors enhance the production of angiogenic factors and down-regulate the expression of anti-angiogenic factors. Many angiogenic factors are upregulated by COX enzymes in the endometrial epithelial cells such as bFGF, VEGF, and angiopiotins. The anti-angiogenic factors such as thrombospondind, angiostatin, and endostatin act as potent vasoconstrictors, and down-regulation of their expression would enhance vasodilatation. Hence, the balance between the expression of the angiogenic and anti-angiogenic factors is essential for normal menstruation, as their balance may determine the degree of permeability and leakage at menstruation. Therefore, the overexpression of COX enzymes in the endometrium of women with HMB may lead to dysregulation in the expression of these factors and imbalance between their function.

Other angiogenic factor such as endothelin, which has strong vasoconstriction property on the smooth muscle cells, is expressed in the endometrial cells including the endothelium. Neutral endopeptidase enzyme has the ability of suppress the endothelin action, hence reduce the permeability. This enzyme is decreased in premenstruation, leading to increase in the endothelin in this time. Increased the enzyme level has been noticed in endometrium in women with HMB, resulting in decline in the endothelin and fragile endometrium.

1.10.1.3 Vascular alternations

Many reports have demonstrated major alternations in the structure of endometrial blood vessels of women with heavy menstrual bleeding. It has been noticed that there is increased in the endothelial cell proliferation in comparison with the endothelial proliferation index observed in normal controls [107]. This may cause alternation in the vascular permeability, which as consequence may affect the availability and accessibility of many endocrine and paracrine factors which have fundamental role in menstruation.

HMB has been associated with increase in PGE_2 levels in endometrium and this increase is greater than that of $PGF_2\alpha$, resulting in decrease in the$PGF_2\alpha$/ PGE_2 ratio[108], resulting in less vasoconstriction of spiral arterioles prior to menstruation. Furthermore, the decline in the endothelin-1, which is a potent vasoconstrictor factor, may result in further deterioration in the constriction of the endometrial blood vessels particularly perimenstruation[109]. It has been noticed that the wall circumference of endometrial blood vessels in women with HMB is larger than that in women with normal menses [110]. In addition, it was noticed that smooth muscle in the endometrial blood vessels walls in women with HMB are less in their proliferation during mid-late secretory phase[111] and maturation than that of the normal controls[112]. This alternation in spiral arterioles maturation may result in further insufficient vasoconstriction and reduced the blood flow resistance; as consequence increase in the menstrual blood loss. All this may delay the endometrial repair due to limitation in the hypoxic insult in the basal layer of the endometrium in women with HMB.

1.10.2 Heavy menstrual bleeding in presence of uterine fibroids

Uterine fibroids are the most common pelvic tumours of the female genital tract. They are benign smooth muscle tumours that arise from the uterine myometrium [113]. Although all women at reproductive age can be affected by fibroids, they largely affect women aged from 40 to 50 years old [114] with an approximate prevalence of 30% [115],and they increase in prevalence to 77% among women in the USA population [116]. Fibroids are particularly common in African women compared to Caucasian women [117] as they have some familial tendencies [118]. However, Baird, et al., 2003 pointed out that the most Caucasian and African women develop fibroids and the difference is that African women develop fibroids at earlier age than white women, have more severe symptoms and a longer period of sustained growth [119]. There may be one single uterine fibroid or multiple fibroids with different size and locations (classification of uterine fibroids is addressed in section 8.1.1). The pathophysiology of these tumours remains unknown, although, there is a hypothesis that each fibroid is derived from a mutation in a single smooth muscle cell [120].

Most fibroids are asymptomatic and are accidently discovered during a clinical or ultrasound examination. However, uterine fibroids can cause severe symptoms in 15-30% of women [115, 121]. Patients with symptomatic fibroids may present with menorrhagia, impairment of fertility, pressure symptoms such as urinary incontinence, constipation and abdominal pain. Despite the benign nature of fibroids, they have a significant level of morbidity depending on the size, anatomic location of the tumour and reproductive status of the women. One of the main symptoms of fibroids is heavy menstrual bleeding (HMB), which takes one of two patterns, increase in the amount of blood loss per cycle or prolonged vaginal bleeding [122]. This symptom can lead to medical issue (iron deficiency anaemia) and social problems present in social embarrassment and improper work life because of the need to change sanitary towels frequently.

In the United States, the uterine fibroids are the first reason for hysterectomies, where 200,000 hysterectomies, 30,000 myomectomies, and thousands of uterine artery embolisation and high intensity focused ultrasound procedures are annually performed to remove or destroy uterine fibroids. In addition, the annual cost of managing uterine fibroids is estimated to be between $5.9-34.4 billion [123].

Although increasing attention to research on this tumour, the pathogenesis of this disorder is not well defined. Many authors have been focused on the initiation of uterine fibroids and how does transformation of normal smooth muscle cell to abnormal smooth muscle cell occur. Furthermore, they have been working to understand the growth of the clinically apparent tumour. Many have studies pointed out that genetic, cellular predisposition, sex steroid hormones and growth factors play an important role in the development and the growth of uterine fibroids.

The cellular origin of uterine fibroids are still not fully understood, but many observations suggested that each fibroid is originated from transformation of a single somatic stem cell of the myometrium under the influence of steroid hormones [124], and earlier studies suggested that uterine fibroids are a monoclonal tumour [113].The histologic composition of uterine fibroids includes disordered smooth- muscle cells submerged in abundant extracellular matrix.

The leiomyoma cells have moderate rate of proliferation and formation of extracellular matrix account an important part of the fibroid expansion.

During the reproductive age of woman, myometrium undergoes multiple cycles of growth changes because of the effect of ovarian hormones or pregnancy hormones. Theses phases of alternation make stem cells more vulnerable to the development of mutations. A single gene mutation such as affecting the gene encoding mediator complex subunit 12 (MED 12), or a chromosomal rearrangement increasing the expression of HMGA2, or other some somatic gene defect, may transform a normal myometrial stem cell to fibroid stem cell, which may have the ability to divide in an uncontrolled manner and develop into mature fibroids tissue [125]. Because of these sequential changing, the mature fibroid cell may acquire epigenetic changes such as DNA methylation and histone modification [126]. In the addition, some cell regulatory markers which play a vital role in fibroid growth such as ß-cathenin and TGF-ß, where changing in their signal pathways can happen by the original single hit, leading to alternation in cell proliferation , survival [127, 128] and formation of extracellular matrix, all this may result in the clonal expansion pattern of stem cells within normal myometrium. Although the majority of cells within this clone differentiate and develop a phenotype similar to that of normal myometrial smooth cells, they still have the characteristic of original mutation or chromosomal rearrangement for supporting further growth [124].

The ability of myometrial tissue to respond to the ovarian hormones for the physiological expansion during the secretory phase of the menstrual cycle or pregnancy may be applied to the fibroid tissue. It has been noticed that most uterine fibroids shrink after menopause, and changing levels of oestrogen and progesterone that associated with the early pregnancy and postpartum period has a great effect on fibroid growth [129, 130]. In addition, GnRH agonists, which decrease the level of circulating ovarian hormones, are able to suppress the growth and reduce the size of uterine fibroids [131, 132]. Theoretically, these observations support the idea of the dependency of uterine fibroids on the ovarian hormones. Researchers who are interested in uterine fibroids noticed a high concentration of oestrogen receptor α and progesterone receptors in fibroid tissue and thought that signal pathway of theses hormones pass through a paracrine mechanism to the stem cells as the later have remarkable low level of

ER and PR with comparison to fibroids and normal myometrial cells. Thus, some authors would consider a class of antiprogestin as the most specific medical approach, which targeting the growth mechanism of fibroids [133-135].

Growth factors can be produced from many cell types but they are mainly secreted from the smooth muscle cells and fibroblast cells. Growth factors have a wide range of biologic effects, and largely act over short distances by either an autocrine or paracrine mechanism. Growth factors are considered crucial factors in controlling cell proliferation and over expression of either the growth factor or its receptor may contribute to tumorigenesis. Growth factors activity is affected mainly by specific binding sites on the surface of the target cells, with subsequent message transmission via signal pathways in the cell.

Several growth factors and their receptors have been recognized in both myometrium and uterine fibroids. TGF- ß consists of three isoforms and it has two types of receptors in myometrial cells TGF- ß I and III. TGF- ß functions as a promoter of mitogenesis and up-regulator to the synthesis of several components of extracellular matrix.

It has been found that TGF- ß3 value is significantly higher in the fibroid cells than the myometrium and TGF- ß3 expression increased in fibroids with no difference in the TGF- ß1 expression. The effect of TGF- ß as stimulator and as inhibitor depends on several factors including, the target cells, the amount of TGF- ß, and presence of other growth regulators. It is though that TGF- ß may act as stimulator for smooth muscle cell proliferation and production of extra-cellular matrix particularly when present in low level (TGF- ß3- TGF- ß1). This finding has been supported by a study that examined cultured smooth cells from the aorta treated by TGF- ß and found stimulation of autocrine secretion of PDGF, revealing the indirect effect of TGF- ß on the proliferation of smooth cells. On the other hand, down regulation of PDGF- R has been found in the presence of a high value of TGF- ß. The regulatory effect of progesterone on TGF- ß emerged when a study observed that unusual increases in TGF- ß3 mRNA levels in the secretory phase relative to the proliferative phase in fibroids. However, there is no difference in the TGF- ß levels in myometrium cells throughout the menstrual cycle. In addition, one study pointed out no significant effect of TGF- ß on the cell proliferation. Important point with regard TGF- ß is that the gene

coding for TGF- ß is located near 14q23-24 the most common chromosomal rearrangement in fibroids.

The majority of the symptomatic patients with fibroids experience heavy menstrual bleeding. There is a strong association between uterine fibroids that destroy the uterine cavity (submucosal fibroids) and heavy menstrual blood loss in comparison with other types of uterine fibroids. However, to our knowledge, there is no estimation of the proportion of women with uterine fibroids who had HMB.Women with HMB are more likely to have fibroids and women with fibroids can be asymptomatic. Furthermore, postmenopausal women with submucosal uterine fibroids and using hormonal therapy were found to have two fold higher risk of abnormal uterine bleeding than women without submucosal fibroids. Therefore, other pathological reasons for abnormal uterine bleeding such as coagulopathies e.g. Von Willebrand's disease as discussed above, should be excluded before attributing HMB to fibroids. On the other hand, others have concluded that only 25% of patients with submucosal fibroids may have heavy menstrual bleeding, revealing uterine fibroids regardless their size or site may have paracrine molecular effects on the adjacent endometrium that are extensive enough to cause HMB [136].

The mechanism by which fibroids cause HMB is unknown. Whereas in the past it was thought that abnormal bleeding associated with fibroids was due to disturbance in the ovarian hormones [137], today it is believed that abnormal vaginal bleeding is because of uterine factors, especially if it is confirmed that no steroid hormones differences have been found between women with fibroids and women without fibroids [138].

Several theories tried to explain the pathophysiology of HMB associated with fibroids. Increase the endometrial surface area because of fibroids is one of aetiological factors that has been considered in the literature. However, no effect of this factor was found on idiopathic HMB, which makes this suggestion controversial [139]. High vascularity of the uterus associated with fibroids is another possibility for the mechanism of HMB. Additionally, recent reports have emerged describing the relation between development of fibroids and dysregulation of several growth factors, or their receptors, leading to vascular

dysfunction and angiogenesis, which contributes to dysregulation of vascular structure in the uterus, subsequent to HMB [122].

Another explanation has taken the interference of fibroids with the normal uterine contractility into account, but the less effect of fibroids on the contractility of blood vessels rejects this suggestion and considers it only as a major reason for dysmenorrhoea associated with fibroids. Compression of fibroids on the venous plexus of the adjacent tissues causes congestion to the myometrium and endometrium lead to abnormal uterine bleeding [140]. Because of the role of prostaglandin PG and endothelin in the rise of HMB make some authors to think about them as factors in the mechanism of HMB with fibroids, particularly when one of these studies used PG synthetase inhibitor as a treatment for HMB and found decrease in the blood loss, but the result was not statistically significant. Endometrium ulceration and endometriosis that associated with fibroids especially the submucosal ones is an additional reason for HMB [141]. However, fibroid women with HMB should be investigated with the aim of excluding any other pathology particularly when HMB is not a feature of fibroids.

The early studies suggest that increases in the endometrial surface could be a reason for heavy menstrual bleeding (HMB) in fibroid uterus; in addition, there is a basic modification in the vascular structures of the fibroid uterus. Alternation in vascular structure is demonstrated by change in venous structures in the endometrium and myometrium resulting in a fragile and engorged plexus (venule ectasia). This venous ectasia may be because the local action of growth factors as consequence of altered synthesis and expression in fibroids. With ectatic venules, the haemostatic actions of the platelet and fibrin block may be plagued by the bigger diameter of the vessels, which causes the ‘flooding’ seen in women with HMB.

A recent explanation of mechanisms of angiogenesis in the fibroid uterus demonstrates local dysregulation of vasoactive growth factors or growth factor receptors in fibroids or fibroids myometrium. Furthermore, abnormalities in arterial structures in fibroid uterus, where fibroids have an increased arterial supply has been proved by some traditional studies. The myometrium is a massive reservoir for paracrine or endocrine factors that regulate endometrial

function and the directional blood flow from the myometrium to the endometrium facilitates this interaction. The research for the association between uterine fibroids and heavy menstrual bleeding has demonstrated a biochemical differences between the fibroid tissue and normal myometrium, including increases in some matrix metalloproteinase [142, 143]and antigenic and growth factors, basic fibroblast growth factor, transforming growth factor-ß, vascular endothelial growth factor as well as plasminogen activators and inhibitors [144].

TGF-ß may have a direct impact on the endometrial haemostasis and the increased levels of TGF-ß in the adjacent fibroids to the endometrium can reach the endometrial stromal cells and lead to reduction in the production of plasminogen activator inhibitor (PAI), anti-thrombin III and thrombomodulin, which play a vital role in the local endometrial haemostasis [136]. Furthermore, TGF-ß plays a role as stimulator of angiogenesis [136] andproduction and differentiation of extracellular matrix[145].

Endometrial haemostasis is a sensitive balanced process between platelet aggregation and fibrin formation from one side and platelet inhibition and fibrinolysis on the other [146]. The plasminogen activator is increased across the proliferative phase and reaches the peak of its level at the mid-cycle, and it declines in the secretory phase and then increases again premenstrually. On the other hand, the plasminogen activator inhibitor is increased by the end of the proliferative phase and through the secretory phase and maximised the level during the menstruation [147, 148].This haemostatic mechanism regulates the blood flow in normal menstrual cycle. The increased levels of plasminogen activator inhibitor expression in fibroid tissue suggest that uterine fibroids may be unable to initiate the fibrinolytic process, leading to prevention of the thrombolysis process in fibroids-associated vessels. In addition, there is a four-fold increase in the expression of PAI-1 as well as a reduction in antithrombin III and thrombomedulin in the endometrium of women with uterine fibroids that is associated with heavy menstrual bleeding [136],thereby; elevation of PAI may contribute to the impaired hemostatic process in women with uterine fibroids, resulting in heavy menstrual bleeding.

The aberrant angiogenesis is associated with the secretion of growth factors such as VEGF, TGF-ß, fibroblast growth factor (FGF)[149]. Dysregulation of angiogenic and regulatory growth factors may further contribute to heavy menstrual

bleeding in women with uterine fibroids and the over-expression of growth factors or their receptors in fibroids uterus particularly heparin binding group of growth factors such as basic fibroblast (bFGF),VEGF, heparin-binding growth factor (HBEGF), platelet-derived growth factor (PDGF) compared to the adjacent normal endometrium could cause abnormal uterine bleeding [144]. However, under-expression of angiogenic inhibitory factors or vasoconstricting factors or their receptors in fibroids may also result in abnormal bleeding. Nevertheless, the exact role of these growth factors in heavy menstrual bleeding associated with uterine fibroids need to be determined.

The expression of both growth factors and their receptors may be regulated by ovarian steroid hormones, as their expression appears to undergo menstrual cycle phase specific up regulation in fibroids tissue compared with normal myometrium. Hence, both receptors of oestrogen and progesterone are over-expressed in fibroids compared with myometrium. There are certain proteins in fibroids which appear to undergo menstrual cycle phase-specific up-regulation in fibroids compared with normal myometrium such as mRNA for collagen types I and epidermal growth factor (EGF) mRNA which the relative overexpression. Growth factors or their receptors that are regulated in different way in fibroids or the endometrium of fibroids uterus potentially mediate fibroid related HMB by acting on vascular tissue and increase proliferation or change vessel calibre. Fibroids with their large extracellular matrix content may be a reservoir for these factors. Both bFGF and VEGF mainly regulate endothelial cell function and hence may promote the endothelial cell migration essential to angiogenesis [149]. Heparin binding growth factors such as heparin-binding EGF-like growth factor (HBEGF) and platelet-derived growth factor (PDGF) largely regulate fibroblast and smooth-muscle cell function and therefore may influence vascular smooth-muscle, fibroid or myometrium, or the endometrial stromal cells.

There appears to be menstrual cycle-specific expression of VEGF in the uterus. During the proliferative phase, VEGF mRNA was detected in different cells in the stroma with weak expression in the glands and was also detected in the myometrium, at the endometrial–myometrial border. In contrast, during the secretory phase there was increasing expression in the glands, with peak expression in the menstrual phase and with the disappearance of stromal expression. In the past, VEGF concentrations were found to be similar in both myometrium and fibroids and to have no significant menstrual cycle variability.

1.11 Treatment of heavy menstrual bleeding

1.11.1 Medical treatment of fibroids

1.11.1.1 Gonadotropin-releasing hormone (GnRH),

Gonadotropin-releasing hormone (GnRH), also known as luteinising-hormone releasing hormone (LHRH), is produced in neurons in the hypothalamus. It is transported via axons to small blood vessels (portal vessels) in the median eminence, where it is released into the blood. The blood vessels are draining the anterior pituitary, allowing GnRH to reach the anterior pituitary in high concentrations. In the pituitary, GnRH acts through the GnRH receptors on the gonadotropic cells to stimulate the release of follicle-stimulating hormone (FSH) and luteinising hormone (LH) to the circulation. The pulsatile secretion pattern of GnRH reflects on the cyclic release of LH and to a lesser extent of FSH.

GnRH agonists are known to be used for short-term (≤ 6 months) or for long-term (>6months), and in order to avoid the side effects of GnRH on bone density, the short-term administration is the most used protocol in the practical field or it is combined with hormonal addback. GnRH agonists have been shown to decrease the size of uterus as well as the size of fibroids [131, 132], and reduce the menstrual blood loss. One possible explanation of the effect of GnRH agonists on both the size of uterus and fibroids appears to be the result of decreased levels of oestrogen and progesterone (P) that are induced by GnRH agonists, however, other mechanisms, including a decrease in the size or number of leiomyoma cells, which may cause induction of fibroid degeneration and hyaline necrosis, a reduction in extracellular matrix, and/or a decrease in blood flow to the uterus, may be important. However, this effect is present only during the therapy duration and stop shortly after stopping it, where uterine fibroids return to the original size within couple of months[150].

It has been noticed that the preoperative use of GnRH agonists may improve preoperative haemoglobin, and reduce operating times and hospital stays, and

allow some cases to have a less invasive operation [151]. There is some evidence that reduction in the size of uterine fibroid makes a higher proportion of fibroids protrude into the uterine cavity, thus it facilitates the resection and short the operating time[152, 153]. Reduction in the operating time is one of the main reasons to adopting preoperative use of GnRH agonists for women having hysteroscopic resection of submucosal fibroids, especially enlarged fibroids, which reduce the risk of excessive fluid absorption and overload. However, there is limited evidence supporting preoperative use of this therapy due to lack of randomised control trials in this concept. Nevertheless, one of the randomised trials does not recommend the routine use of preoperative GnRH therapy for hysteroscopic resection of fibroids as no significant benefit was observed between cases and controls [154].

1.11.1.2 Evonorgestrel intrauterine system (Mirena IUS)

Evonorgestrel is a progestin, named levonorgestrel because it is the levorotatory form of norgestrel. The levonorgestrel-releasing intrauterine system has been available since the mid-1970s. It is marketed under the name Mirena. Mirena IUS is a safe and accepTable form of contraception, providing highly effective contraception for up to five years. It also has a range of noncontraceptive usage including treatment for heavy menstrual bleeding, endometriosis, and endometrial hyperplasia. The Mirena IUS has also been used in combination with estrogen for hormone replacement therapy and as an alternative to hysterectomy. Mirena IUS is planned to provide an initial release rate of 20 microgram (µg) per day of levonorgestrel. This decreases to 11 µg per day by the end of five years, with an average release rate of 14 µg per day during the life of the Mirena IUS. Levonorgestrel-releasing intrauterine system consists of a T-shaped polyethylene frame with a steroid reservoir around the vertical stem. The reservoir consists of a white or nearly white cylinder, made of a levonorgestrel, containing a total of 52 mg levonorgestrel and covered by a semi-opaque silicone (polydimethylsiloxane) membrane. The T-body is 32 mm in both the horizontal and vertical directions. The polyethylene of the T-body is compounded with barium sulfate, which makes it radiopaque. A monofilament brown polyethylene removal thread is attached to a loop at the end of the vertical stem of the T-body.

Levonorgestrel works by thickening the cervical mucosa and suppressing the endometrium proliferation. Additionally it produces thin endometrium with fragile blood vessels which imperfect for implantation. On the other hand, the absorbed level of progestin is below the required level to inhibit the ovulation; therefore, most women on this treatment still have regular ovulation. Mirena IUS is indicated for the treatment of heavy menstrual bleeding in women who choose to use intrauterine contraception as their method of contraception. In women with idiopathic heavy menstrual bleeding, the levonorgestrel-releasing intrauterine system reduces menstrual blood loss more effectively than cyclical norethisterone (for 21 days) as a treatment for heavy menstrual bleeding. In comparison of levonorgestrel-releasing intrauterine system efficacy with established medical and surgical treatments, as a part of comparative and non-comparative studies, it was found that this therapy reduce blood loss by 79-97%. However, many gynaecologists advise this therapy for heavy menstrual bleeding in women with fibroids, where there is no a clear evidence about the effectiveness in case of uterine fibroids. There are a variety of medical treatments available for heavy menstrual bleeding, including prostaglandin synthetase inhibitors (non-steroidal anti-inflammatory drugs, NIASDs), antifibrinolytic agents (tranexamic acid) and oral contraceptive pills. Progestogens is used in different forms including, oral, implant, and coil (the Mirena coil), but they appear to be less effective where fibroids are the cause of symptoms.

Therefore, the effect of these medications needs to be examined in case of heavy menstrual bleeding because of fibroids. The following survey assessed the gynaecologists' beliefs regarding the treatment of fibroids particularly when women present with heavy menstrual bleeding in the UK and Libya.

1.11.1.3 Selective progesterone receptor modulators (SPRMs)

Introduction of progesterone to the clinical field has provided a great help in controlling various reproductive function. Many progesterone receptors antagonists have been widely used in fertility control and hormonal therapy. Selective progesterone receptor modulators have been developed sine late 1970s when mifepristone was first prescribed. Selective progesterone receptor modulators are one of the progesterone receptors ligands that synthesised in

three different types including selective progesterone agonist, antagonists, and mixed (agonist/antagonist),which effect on many progesterone target tissue [155].Mifepristone has unique major antagonist properties allowing its use for pregnancy termination. Ulipristal acetate has been used for emergency contraception and has been recently authorised for preoperative uterine fibroid treatment [156]. Further perspectives for SPRMs use include long term estrogen free contraception. However, long term applications will be possible only after confirmation of endometrial safety[157].

Using SPRMs have shown benefit in inducing amenorrhoea, endometriosis associated pain, and reduce the size of uterine fibroids. a number of double-blind, randomized, placebo-controlled trials have demonstrated the efficacy of asoprisnil, mifepristone, telapristone acetate, and ulipristal acetate in reducing uterine fibroids and uterine volume, and suppressing bleeding in women with uterine fibroids[158]. Asoprinil is a SPRM with high binding affinity to progesterone receptor (PR), and no binding affinity to oestrogen receptor, and thought to induce amenorrhoea through anti-proliferative effect on the endometrial tissue throughout the menstrual cycle [159]. However, the mechanism by which SPRMs induce amenorrhoea is still not fully understood. Although observation that agonist activity of SPRM prevents endometrial proliferation may suggest future use of these agents in prevention of endometrial hyperplasia. In addition, other future applications may include endometriosis, endometrial cancer, Cushing's disease, Alzheimer disease or long-term contraception, are currently in development[158, 160].

1.11.2 Surgical intervention

1.11.2.1 Laparotomies

Removal of uterine fibroids through abdominal incision only with conserving the uterus called open myomectomy, and although myomectomy was performed a long time ago (1845), hysterectomy has been the main treatment for women who present with symptomatic uterine fibroids for many years because of bleeding associated with myomectomy. However, women who undergo hysterectomy for very large uterine fibroids have been shown to have an increased risk of blood loss with increasing uterine size. Later on, and because

of haemostatic and surgical technique development, abdominal myomectomy has been used as a conservative treatment for uterine fibroids, and there may be less risk of intraoperative injury with myomectomy when compared with hysterectomy. In addition, myomectomy may be considered for women with large uterine fibroids or difficult fibroids associated symptoms who desire to retain their uterus and desire future fertility. Currently, however, there is no significant evidence regarding the benefit of myomectomy on reproductive outcome as treatment for all types of uterine fibroids, for instance, [161]. Myomectomy has been found to relieve symptoms in 80% of women; however, unrelieved symptoms and /or the need for repeating the procedure over time are frequently reported with this procedure. Therefore, hysterectomy is only the definitive treatment of uterine fibroid.

1.11.2.2 Laparoscopy

Since 1990 laparoscopic myomectomy has provided an alternative to laparotomy when subserosal (since1970s) then intramural fibroid is to be managed surgically. However, pros and cons of this procedure as treatment of uterine fibroids particularly for women with subfertility is still the subject of debate. Laparoscopic myomectomy offers the possibility of a minimally invasive approach to treat subserosal and intramural fibroids by surgery when there are only a small number of them. When this procedure is conducted by experienced surgeons, the risk of peri-operative complications is reduced in particular, the bleeding risk associated with myomectomy. In addition, the procedure may have less post-operative risk of adhesions as compared with laparotomy, and spontaneous uterine rupture seems to be rare after laparoscopic myomectomy, but further studies are needed to obtain clear evidence about the strength of the hysterotomy scars after this technique in comparison with that obtained after laparotomy[162, 163]. Nevertheless, the risk of recurrence seems to be higher after laparoscopic than after open myomectomy. Many studies were launched to compare between the effect of laparoscopic myomectomy and the effect of open myomectomy on the reproductive outcome, and up to date, there is not a certain conclusion regarding the benefit of either of these methods. This uncertainty in the conclusion is most properly because of a lack in the randomised control trials with this at the end point [161]. On the other hand, two randomised control trial compared laparoscopic to open myomectomy, and

demonstrated no significant evidence of the superiority of laparoscopic myomectomy regarding the live birth rate, clinical pregnancy rate, caesarean sections rate, miscarriage rate[164, 165]. However, analyses and interpretation of this data should take into account that these studies are small number studies.

1.11.2.3 Hysteroscopy

Hysteroscopic myomectomy was introduced to the gynaecology clinical practice as result of development of the urological resectoscope, with instrumental modification since 1980s. Nowadays, hysteroscopic myomectomy is the treatment of choice for submucosal fibroids associated with heavy bleeding, and reproductive issue. Hysteroscopic myomectomy has been shown to be safe and effective in the control of menstrual disorders, and in improving the fertility rate [166]. However, no evidence on beneficial effect of the procedure on fertility rates for submucosal G1 and intramural fibroids. The selection of hysteroscopic technique is mainly dependent on the intramural extension of the fibroid- on one hand, and on personal experience and available equipment on the other [167]. Hysteroscopic resection by slicing technique still represents the main procedure for treating submucosal fibroids and about 40% of hysteroscopy procedure are performed under anaesthesia [168], even though, out-patient two step hysteroscopy has been proposed.

The pre-operative use of GnRH has shown to improve hysteroscopy visibility and reduce blood loss during operation [169]. Most techniques aim at the transformation of an intramural fibroid into a totally intra-cavity lesion, thus avoiding a deep cut into the myometrium. The hysteroscopic resection of submucosal fibroids is a safe and highly effective long-term therapy for carefully selected women presenting with abnormal uterine bleeding and fertility problems, where it increases the odds ratio of pregnancy rate in women with unexplained infertility, however, the evidence is still inconclusive[170]. In one randomised study, the odds ratio for pregnancy was 1.88, 2.04, and 3.24 after hysteroscpic myomectomy for submucosal, intramural, and combined submucosal and intramural respectively, and the odds ratio of miscarriage in the same study was 0.63 after myomectomy for submucosal fibroids and 0.89 after intramural and 0.50 after combined submucosal and intramural [171].

1.11.2.4 Vaginal myomectomy

Although vaginal myomectomy was identified for the first time in the middle of the 20th century, this procedure has not been widely adopted. Vaginal myomectomy, in well-selected cases, is feasible, well tolerated technique, and associated with short operating and recovery time [172]. This procedure isthought to be useful even in cases of large, numerous, and intramural fibroids and allows optimal uterine wall reconstruction with minimal tissue trauma. When uterine fibroids are large, numerous and intramural, laparoscopic myomectomy would not be easy unless it is performed by a skilled surgeon. Vaginal myomectomy is more effective to avoid the abdominal trauma and have better operative field than laparoscopic and hysteroscopic procedure, in particularly for large intramural fibroids. In addition, it is not constrained by limitations such as location, size and number of fibroids. Furthermore, vaginal myomectomy has a shorter operating time than laparoscopic myomectomy, whilstothers find vaginal myomectomy equal in operation time to laparoscopic myomectomy. Additionally, another author pointed out that vaginal myomectomy had significantly longer hospitalisation and gas and bowel recovery time and more transient high fevers, but the difference in blood loss was not significant in some findings and significantly shorter in others[173]. Lack of high quality studies and absence of systemic review is the main reason that the controversies are still existed in the evaluation of vaginal myomectomy and laparoscopic myomectomy.

1.11.2.5 Endometrial ablation

Endometrial ablation (EA) is a destruction technique that targets the endothelial surface of the uterine cavity in order to destroy and remove endometrial tissue.This procedure was introduced to the clinical field in Germany in the 1930s, and it became more popular in the latter part of the 20th century. In 1995 endometrial ablation started to be performed under the hysteroscopy vision in the UK, and helped to decrease the number of hysterectomies for HMB by 64% between 1995-2002 [174]. The initial performance of this destructiove method was by using electrocautery through loop and rollerball (80%), laser (18%), and radiofrequency (2%)[175]. The most recent data from the UK (2004-2006) shows that 60% of surgical methods were used for women with HMB in the

premenapousal age [176]. However, the first generation method had minimal impact on hysterectomies in the UK [177]. Currently, EA can be performed under endoscopic direction with the neodymium:yttrium alumnum garnet laser(rarely used), with radiofrequency resectoscope, or with an expanding array of nonresectoscopic EA systems. Obviously, most but not all of the complications associated with resectoscopic endometrial ablation are eliminated with nonresectoscopic endometrial ablation. Many studies and trials in the literature assessed the sufficiency of endometrial ablation for heavy menstrual bleeding, and evaluated the long term outcome for this method. In addition, large number of studies compared between the first and second generation of endometrial ablation regarding the technique itself and the long outcome. There was no conclusive results up to date, however, no evidence of significant difference about the long term outcome of both generation of EA, and less perioperative complications and shorter time operation were associated with the second generation. On the other hand, more technical failure was noticed with the second generation [178] The procedure was originally designed as a less invasive alternative to hysterectomy for the symptom of heavy menstrual bleeding unrelated to structural pathology of the uterus, which was not responsive to medical therapy.

More recently it has become apparent that the procedure can be performed in the presence of submucosal fibroids, with limitation in number of size and location criteria or combined with hysteroscopic resection. However, serious morbidity has been reported with all of the newer systems to date. Long term satisfaction among the majority of well-selected patients treated in clinical trials, but repeat surgery, usually hysterectomy, is performed in 25% to 40% by 5 years after surgery. Some authors thought that moving the procedure to out-patient sittings may enhance its efficiencies.

1.11.3 Less invasive intervention

1.11.3.1 Uterine Artery Embolisation

Uterine artery embolisation (UAE) is a relatively new procedure by which transcatheter bilateral injection of small particles is performed through the femoral arteries to block the uterine arteries. Since 1995, this procedure has

been offered as alternative therapy to surgical interventions for women with symptomatic uterine fibroid particularly associated with HMB [179]. Although there is a concern regarding the post- procedure pain, post embolisation syndrome, reduced ovarian reserve and early menopause, UAE seems to have less complications in comparison with other surgical technique, and appeals to women who desire to avoid major surgery and keep the uterus. Nevertheless, there is uncertainty about the effect of UAE on fertility and on pregnancy outcome [180], although live births were recorded [181, 182]. Hence, women with fibroids who desire future fertility need carful counselling. Notably, there is a good rate of patient satisfaction after long term follow up in women with HMB, with no significant difference compared with other surgical methods. In addition, there was no difference between the UAE and myomectomy regarding major complication. However, the later procedure reported better fertility rate than UAE, but the evidence was not appreciated because of low cohort study [183]. Also a higher rate of new fibroids formation was identified in women underwent myomectomies compared to UAE after 5 years evaluation [184]. On the other hand, reintervention rate was higher among women who had UAE compared no reintervention after myomectomies.

1.11.3.2 Magnetic-resonance guided focused ultrasound surgery

Magnetic-resonance-guided focused ultrasound surgery (MRgFUS) is a non-invasive thermo-ablative technique that uses focused high-energy ultrasound to remove fibroid tissue. As in the usual diagnostic ultrasound, the ultrasound waves pass through the anterior abdominal wall. Significant heating only occurs where the waves converge at the focus. Magnetic resonance guidance provides continuous imaging of the fibroid and other vital structures such as bowel, bladder and sacral nerves.

MRI scans identify the tissue in the body to be treated and are used to plan each patient's procedure. MRI provides a three-dimensional view of the targeted tissue, allowing for precise focusing and delivery of the ultrasound energy. MRI also enables the radiologist to monitor tissue temperature in real-time to ensure adequate but safe heating of the target. Immediate imaging of the treated area

following MRGFU helps the radiologist determine if the treatment was successful. MRgFUS was approved by the US Food and Drug Administration in 2004, while NICE recommended that the procedure should be used in an audit and research setting. Significant improvement in quality-of-life parameters has been reported in women undergoing MRgFUS. MRgFUS is safe and effective treatment for uterine fibroids [185]. There are no urgent surgical procedures or bowel injuries, which are reported in many early studies of new investigational devices for uterine fibroids. Furthermore, women who have treatment with MRgFUS do not appear to have post-embolisation syndrome symptoms that associated with UAE. Patient undergoing MRgFUS usually has short recovery time and return to work after approximately 1 day, compared with 10-13 days after UAE and approximately 6 weeks after open myomectomy or 8weeks after hysterectomy. However, a proportion of further treatment was reported [185].

MRgFUS can be considered as minimal invasive procedure alternative to other surgical and radiological procedure for treating uterine fibroids that improve quality of life and fertility. However, other report pointed out better score of quality of life and low reintervention rate recorded with UAE than MRgFUS [186]. Pregnancies have been reported and their outcome seems to be similar to that of age matched women who have fibroids but no treatment (). In addition, greater experience, device improvement, and availability to larger number of patients is required to increase the outcome of the procedure [187]. Where there are limited centres in the US (20 centres) and only 2 units in the Uk.Furthermore, the position of MRgFUS as treatment of fibroid need to be established in comparison with the other available treatment modalities by means of randomized controlled clinical trials.

1.11.3.3 Myolysis

Laparoscopic myolysis was introduced as a minimally invasive technique for the treatment of fibroids in the late 1980s. The procedure action aims to destroy the fibroids tissue using a number of focused energy delivery systems such as supercooled cryoprobes, radiofrequency electricity devices and focused ultrasound monitored by real time magnetic resonance imaging. These different energy sources are delivered by a laparoscope and in some instances by a hysteroscopy technique for both thermomyolysis and cryomyolysis. In the

cryomyolysis technique, a cryoprobe is inserted into the centre of the fibroid, and tissue ablation is achieved by creating an iceball within the fibroids, repeated freeze/ thaw cycles are needed to achieve the desired effect. A reduction in the fibroids size after cryomyolysis is poorly achieved comparing to other myolysis techniques. In addition, it might lead to serious renal failure as a result of the release of myoglobin. The thermomyolysis therapy is designed to transmit heat into the target tissue causing coagulative necrosis, which may be suitable for large size fibroids,while maintaining three dimensional control of energy delivery to thermally destroy the target volume. Laparoscopic myolysis may be effective in aggravating fibroids shrinkage, with a decrease in size of fibroids and some decline in the uterine size as well [188], and this technique can be applied as an alternative to laparoscopic myomectomy in selected patients for instance women with large and multiple intramural fibroids, patients who approaching the menopause aged , patient over 40 years old age or those who completed their family [189-191]. However, the technique should be assessed in a prospective randomized controlled trial versus other minimally invasive techniques as evidence regarding the procedure based on case series [188].

1.12 Hypothesis and aim

Normal menstruation is a result of interaction between inflammatory markers, antigenic factors and the endocrine system. The causes of menstrual abnormalities include heavy menstrual bleeding (HMB) and are not fully understood. However, it is recognised that local cellular and molecular signalling events are implicated in changes in the endometrium physiology and lead to HMB.

Uterine fibroid is fibromuscular benign tumour in the female reproductive tract during child bearing age. It affects a wide range of women throughout the world. The most presenting symptom of fibroids is menstrual abnormalities, including heavy menstrual bleeding. However, the mechanism by which fibroids lead to HMB is not known and there is no group studied it yet.

This thesis is based on the hypothesis that uterine fibroid is contributed to HMB in different ways that may happen in absence of any uterine pathology.

The aims of the project were to:

- Estimate the role of endometrial physiology in the aetiology of heavy menstrual bleeding in women with uterine fibroids

- To evaluate the role of COX, PG, VEGF and inflammatory factors (IL8) in this process

- To determine whether these factors act differently in women with uterine fibroids compared with those showing no uterine pathology.

Chapter 2:

2. Materials and methods

2.1 Subjects

The subjects in this study were women of reproductive age who had regular menstrual cycles lasting between 21 and 35 days. Biopsy specimens were collected from 79 women presented with the complaint of heavy menstrual bleeding (HMB). Some of these women had uterine fibroids and they were recruited from the hysteroscopy clinic (Stobhill hospital, Glasgow) and intervention radiology clinic (Gartnavel hospital) (group 1, G1, n=22). Others underwent hysterectomy as treatment for their uterine fibroids (group 2, G2, n=16). Uterine fibroids in all participants were diagnosed by ultrasonography or magnetic resonance imaging (MRI). In group two (G2),there were 41 women who had no evidence of uterine abnormality but who had presented to the hysteroscopy clinic with HMB (comparative group) (Table 2.1&Figure 2.1). The study protocol was approved by the ethics committee West of Scotland REC 5, REC reference number (10/S1001/1).

Participants filled in a questionnaire obtaining their demographic and clinical data (see Appendix 1), including, menstrual history, gynaecological and obstetric history as well as drug history. Subjects completed the Pictorial Blood Assessment Chart (PBAC) at least during one period[192]. All women had to sign a consent form. The subject's details will be addressed in the results section of each chapter.

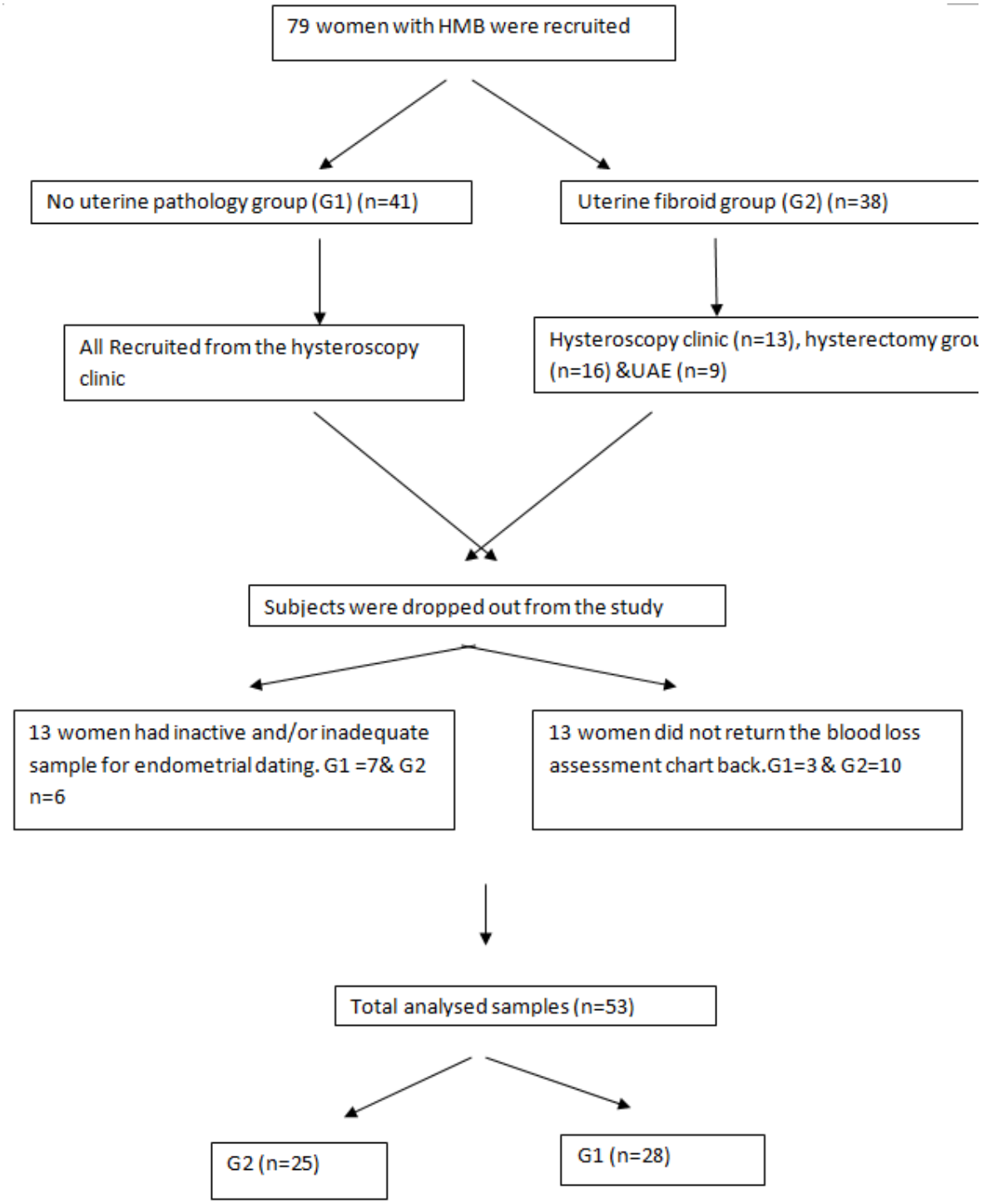


Figure 2.1: summary of the recruited subjects in the study, including drop out subjects and final analysed number of subjects samples in each group.

Table 2.1: Summary of demographic details of participants

The character	Fibroid group(G1) N=45	Normal uteri group(G2) N=30	P value
Age	45.72±0.93	44.83±0.79	0.47
BMI	27.98±0.87	29.50±1.2	0.33
Length of period			
<3 days	N=0	N=3	
3-5	N=10	N=3	
5-7	N=15	N=15	
7-10	N=12	N=7	
>10	N=8	N=2	
Hormonal assays (mean±SE):			
LH (mIU/mL)	3.95±0.72	6.2±(2.2)	0.33
FST(mIU/mL	9.5±2.5	9.2±2.8	0.95
E2 (pmol/L	333±66	435±139	0.51
Progesterone (nmol/L)	9.6±2.8	9.8±3.3	0.97
Types of uterine fibroids:			
Submucosal	(n= 12)		
Intramural	(n= 17)		
Subserosal	(n=7)		
multiple types of fibroids	(n=9)		
Excluded biopsies:			
No PBAC was provided	(n=3)	(n=10)	
Not adequate for endometrial staging	(n=3)	(n=1)	
Inactive endometrium	(n=4)	(n=5)	
Endometrial staging:			
Proliferative	(n=10)	(n=8)	
Early secretory	(n=8)	(n=4)	
Mid-secretory	(n=4)	(n=5)	
Late secretory	(n=2)	(n=1)	

2.2 Histological Studies

2.2.1 Biopsy specimen- Collection

2.2.1.1 Biopsy specimen- Collection- Uterine biopsies

2.2.1.1.1 Group one

Endometrium was collected by means of a Pipelle sampler from women who attended the hysteroscopy clinic in Stobhill hospital to investigate their heavy menstrual bleeding and from women who underwent uterine artery embolisation. In addition, uterine biopsies including,endometrium , myometrium and fibroidstissue, were collected from fresh hysterectomisedfibroid uteri specimen by a pathologists in the pathology laparatory, Glasgow Royal Infirmary hospital (GRI). The endometrial biopsieswere collected at the time of hysteroscopy during the first attendance and at the time of Uterine Artery Embolisation (UAE) which performed in the radiology department, Glasgow Gartnavel hospital. All hysterectomy operations were performed in the gynaecology department, GRI.

2.2.1.1.2 Group two

In G2, Endometrium was collected by means of a Pipelle sampler from women who attended the hysteroscopy clinic in Stobhill hospital to investigate their heavy menstrual bleeding with no evidence of uterine pathology.

2.2.1.2 Biopsy specimen -Collection- Blood samples

A five millilitre blood sample was obtained in a lithium heparin bottle at the time of having the uterine biopsies (endometrial, myometrium and fibroid tissue) from all participants for hormonal assessment including estradiol, progesterone, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH).

In addition, serum was collected for the measurement of cytokines: tumour necrotising factor-α (TNF-α), transforming growth factor-β(TGF-β), interleukin 1,6 and 8(IL1, 6, and 8) and c reactive protein CRP), Proangiogenic (VEFG A) and intercellular adhesion molecule -1(ICAM-1).

2.2.2 **Biopsy specimen -Handling and storage**

2.2.2.1 Biopsy specimen -Handling and storage-uterine biopsies

In both G1 and G3 ,every Pipelle endometrial biopsy was divided immediately into two parts, where one part was put in formalin and sent to the pathology department in the Glasgow Royal Infirmary hospital (GRI) for paraffin embedded block process. The second part was put directly in RNA later solution, Ambion R 0901 RNA Later® (0.5-1 cm of tissue sample in 5-10 ml of the solution respectively) and stored in -20C° freezer (Reproductive and Maternal Medicine laboratory. McGregor building, 2nd floor, Glasgow Western Infirmary). After two weeks, biopsies were transferred to a cry-pot by using liquid nitrogen and stored in -80 C° till used. Note, when there was not enough pipelle sample obtained the whole tissue was sent for histopathology assessment and paraffin block process. RNA was then extracted from the blocks (see below).

In G2, the pathologist was informed in advance about the arrival of the specimen and during the hysterectomy the excised fibroid uterus was taken immediately (within 5 minutes) to the pathology department GRI where biopsies from endometrium, myometrium and from each type of fibroid(submucous, intramural and subserosal) were taken by the pathologist. The biopsies were put in RNA later within a maximum of 10 minutes from removal of the uterus and they were stored in same manner as above.

2.2.2.2 Biopsy specimen -Handling and storage- blood samples

Blood samples were centrifuged at 13000 rpm for 15minutes in temperature 4°C. The serum was put in 6 tubes (0.5ml in each tube) then 3 tubes were stored in each of two different -80C° freezers. 1 ml serum from each sample was sent to the biochemistry department-GRI where estradiol, progeterone, Luteinizing

Hormone (LH) and Follicular stimulating hormone (FSH) assays were assessed using ARCHITECT reagent for each assay through the ARCHITECT í System.

2.3 Blood loss assessment

The Pictorial Blood Loss Assessment Chart (PBAC) was used to estimate how much blood women lose during their periods. This is an internationally validated chart[192]. Participants had the chart with them at home when they attended the hysteroscopy clinic to fill in when they had their period. For participants who were on the list for hysterectomy or uterine artery embolisation,the pictorial chart was sent to them in advance to complete during their periods. Women indicated the degree to which the sanitary wear was soiled by indicating the number of slightly, moderately and heavily soiled pads and tampons that they used. Assessment of blood loss using this chart and the results from this study are detailed in Chapter 3.

2.4 Histological dating

Histological dating was carried out by Professor Williams, University of Edinburgh according to Noyes et. al.[193],to confirm the stage of the menstrual cycle. All histological stages were consistent with patient's last menstrual period (LMP) and circulating estradiol and progesterone concentrations in venous blood obtained was collected at the time of tissue collection. Consistency between these three parameters has been approved to be robust method for characterising endometrial samples [194]. Endometrial samples where there was not enough for dating the endometrium were excluded from our analysis.

2.5 Real Time quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The standard PCR a laboratory method that allows exponential amplification of a targeted DNA molecule, then the amplified DNA is detected at the end of the reaction which can be time consuming. The real time quantitative PCR follows

the general principle of the PCR technique, however, reverse transcriptase enzyme is used to generate complementary DNA from mRNA. In addition, the amplified DNA is detected as the reaction progresses in real time. Because of the simultaneous amplification and detection, quantification is performed once per cycle (Figure 2.1). This quantity can be either an absolute number of copies or a relative amount when normalised to DNA input or additional normalising genes.

Figure 2.2: Shows summary of processes involved in real time PCR

2.5.1 **RNA extraction**

RNA isolation was performed in a biosafety cold hood after all work surfaceshad been cleaned up using either 75% Ethanol or Zap RNase and all other equipment such as pipettes, forceps and the homogenizer were also carefully cleaned to prevent contamination.

2.5.1.1 *Isolation*of RNA from frozen samples

Tissue samples were kept frozen on dry ice while weight measurement was taken. The balance was zeroed with cold cryo-pot on it and quickly the tissue sample was added to the pot and weighed. The weight of samples taken was between 50 and 100mg, large pieces of sample were ground into smaller pieces using a cold mortar, spatula and scrape and adding liquid nitrogen some ties to keep them cold. Every sample was transferred to a glass tube containing Trizol solution (1ml of Trizol was added to 50-100mg of tissue sample), then were homogenized using a hand held homogenizer and separated into 1ml aliquots in autoclaved 1.5 eppendorf tubes. The tubes were incubated at room temperature (RT) for 5 minutes to permit complete dissociation of nucleoprotein complexes. 0.2ml of chloroform were added to each 1ml Trizol and shaken vigorously in

order not to reduce the RNA yields. Tubes were incubated for 3 minutes at room temperature before they were centrifuged at 13,000 rpm for 15 minutes at 4°C.The upper aqueous phase was transferred to fresh autoclaved tubes and 0.5ml of Isopropyl alcohol was added to each 1ml Trizol used in the initial homogenization to precipitate RNA from the aqueous phase. A second centrifugation was performed at 13,000 rpm for 10 minutes at 4°C after tubes were incubated for 10 minutes at room temperature. The isopropyl alcohol was decanted and a small white pellet was left at the bottom of the tube. 1ml of 75% of ethanol was added to each tube then vortexed. At this stage some sample was stored in - 80C°and the rest of the protocolcontinued later on. The third centrifugation was done at 10,000 for 5 minutes at 4°C. Ethanol was poured off and the tubes tapped carefully in order not to dislodge the pellet and left to dry for 10 minutes at room temperature. Appropriate amounts of Diethylpyrocarbonate (DEPC) treated water were added to each tube according to the pellet size (range between 40µl for a large pellet and 15 µl for the smallest one). Tubes were then vortexed, centrifuged quickly and incubated twice for 5 minutes at 65°C in heating block. Tubes kept on ice all the time during Nanodrop measurement and then stored at -80C°.

2.5.1.2 Isolation of RNA from formalin –fixed paraffin- embedded samples

Extraction of RNA from formalin-fixed paraffin- embedded tissue (FFPE) was required for some samples where there was no fresh stored tissue. Many protocols have been described to extract RNA from FFPE samples. Most of these protocols follow similar basic principles and the RNA is extracted by spin column purification according to: deparaffinization, followed by cell disruption with heated proteinase K, then RNA isolation by alcohol precipitation [195].
I used Ambien kit 1975 (Recover All total nucleic acid isolation kit-Life technologies, Paisley, UK) and followed the protocol as directed. I practiced the method at the Molecular Pathology, Pathology Department-GRI supervised by Dr David Millen.
A histological section of 10-µm thickness was cut from each block using a conventional microtome. A separate blade was used for each block to prevent contamination. The section was collected by cleaned forceps in an Eppendorf tube (I worked on 2-3 tubes at a time). The section was deparaffinazed by 1 ml

of xylene added to the tube, mixed then centrifuged at 10,000rpm for 5 minutes at 4°C, followed by repeated addition of 1 ml absolute alcohol to the pellet. It was vortexed then centrifuged at full speed for 5 minutes. Alcohol was aspirated and discarded without disturbing the pellet. The pellet was allowed to air dry at room temperature for 10minutes before the addition of lysis buffer. 400µl of digestion buffer and 4µl of protease were added to the samples (from the kit). The tubes were mixed gently, then placed into the rotatory oven at 50°C and incubated with rotation for 3 hours. Proteinase K incubation at high temperature allows efficient degradation of proteins that have cross linked with each other and RNA. . In the most recent protocol proteinase K incubation for 15 minutes only is effective, but I preferred to use the longer incubation as it was assessed and recommended by people working in the Molecular Pathology, Glasgow Royal Infirmary GRI.After proteinase K incubation, RNA was isolated by quantification and qualification of RNA by adding 480µl additive solution from the Ambion kit. The solution should be white and cloudy after mixing, and then it turned clear by addingabsolute alcohol to each tube. At this stage, a filter cartridge was placed into one of the collection tubes supplied in the kit.

The mixed solution was pipetted into the filter cartridge and centrifuged at 10,000 rpm for 60 seconds to pass the mixture through the filter then the flow through solution was discarded. The filter cartridge was washed by 700µl and 500µl of wash 1 and wash 2/3 solution respectively. DNase mix was added to the centre of the filter, where the mix including DNase buffer 6µl, DNase 4µl and nuclease free water 50µl. A master mix was made when more than one sample was processed.

The washing process was repeated after 30 minutes incubation at RT once with 500µl of wash 1 and washed twice with 500µl wash 2/3. The filter cartridge was transferred to a fresh collection tube and 15µl of nuclease free water, heated to 95°C, added to the centre of the filter. The tube was incubated for 60 seconds at room temperature then centrifuged for 1 minute at 13,000 rpm to pass the mixture through. The collected sample volume was checked by the Nanodrop and stored at -80°C.

2.5.2 **RNA quantification and qualification**

The quantity of total RNA extracted above was examined by using an automated spectrophotometer (RNA 6000 Nonodrop). The concentration of total RNA in an aliquot of 1µl of sample was determined (ug/ul) and the ratio of optical density at a wavelength of 260nm to a wavelength of 280nm (260:280) was additionally calculated. The sample was considered not to have sufficient purity for use in further work when it had 260:280 ratios less than 1.6. Quality and integrity of the extracted RNA was examined by Agilent 2100 Bioanalyser system. This was as a paid service from the Biochemistry Department, University of Glasgow (Glasgow Biomedical Research Building). Only RNA that displayed intact 18Sand 28S peaks was reverse transcribed (RT) to cDNA for real-time PCR analysis. RNA integrity number (RIN) for the tested samples with clear 18S, 28S peaks was between 3 to9.
After about half of our samples had been analysed by Agilent bioanalysis, I noticed that Nanodrop measurement results in addition to Ct values of endogenous control only appeared to be a good indicator for the purity of RNA samples that were used for the Taquman technique.In contrast to the microarray method, which needs high RNA Integrity Number (RIN) (≥ 7); there is no a clear cut-off point ofRINis required for Taquman process. Therefore, usable RNA samples may be discarded as poor quality because of low RIN. However, using the endogenous control values to evaluate the quality of extracted RNA is a detection sensitivity way as it requires significant hands-on time to complete loading and preparation of TaqMan plate.

2.5.3 **Quality of RNA yield from paraffin versus RNA yield from frozen samples**

Four samples were used to compare RNA extraction from frozen paraffin embedded tissue. The quantity of RNA recovered from the FFPE samples was lower than that recovered from frozen samples at approximately 60 ng/µl from FFPE compared to approximately 1200 ng/µl from frozen samples. This translated to lower absolute quantities of the mRNAs of interest as indicated by higher CT values compared to those from the frozen samples. The mean B-actin CT from FFPE samples was 36, versus 26 from frozen samples. This potentially

indicates more RNA degradation from the FFPE samples compared to that from the frozen samples.Delta CT values, which reflect on the relative mRNA expression, do not appear to have been affected by the lower RNA yield, as there is no significant difference between delta CT in the frozen compared to the FFPE samples (p= 0.67) (Figure2.2).

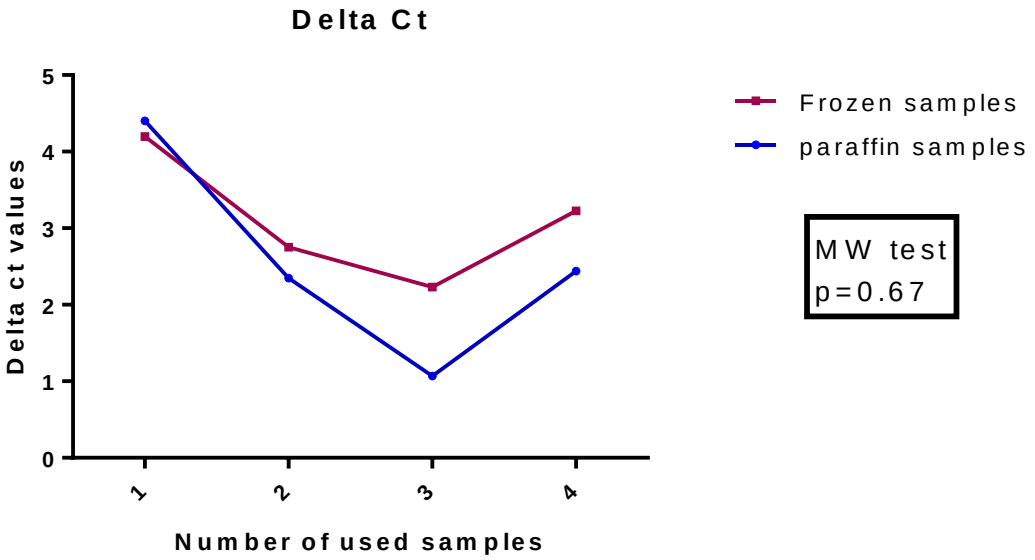


Figure 2.3: Delta CT values for a vascular marker obtained from frozen and paraffin embedded samples.

2.5.4 Generation of complementary DNA (cDNA)

Quantification of gene expression was achieved using the sample of mRNA extracted above. Batches of 30 µl of cDNA was synthesised from approximately 5µg of total RNA from each sample using the High Capacity cDNA Reverse Transcription kit(Life technologies, Paisley, UK) according to manufacturer's protocol.

Each time a patch of cDNA was made, a simultaneous control experiment was performed on a sample of 'spare' total RNA. The master mix for the control

sample excluded the multiscribe reverse transcriptase (MRT), and therefore it acted as negative control run for each 96 well TaqMan plate (Table 2.2).

Table 2.2: Content of TaquMan master mix and No RT.

Preparations	Sample volume of mastermix	No RT mastermix
10xRT Buffer	1ul	1ul
25xdNTPs	0.4ul	0.4ul
10xrandom primers	1ul	1ul
Multiscribe reverse transcriptase	0.5ul	0
Superasin (1U/ul)	0.5ul	0.5ul
Nuclease free water	1.6ul	2.1ul
Total per reaction	5.0ul	5.0ul
Total volume	30 ul(15.0ul of mastermix+15.0ul of RNA sample)	10ul(5.0ul+5.0ul of RNA sample)

All primer probes used for gene expression quantification such as human ACTB (B actin), cyclooxygenase 1 (COX-1), cyclooxygenase 2(COX-2), interleukin8 (IL8), vascular endothelial growth factor-A (VGEF-A), vascular endothelial growth factor receptor 1(VEGFR1) and vascular endothelia growth factor receptor 2(VEGFR2) were purchased as predesigned, inventoried 'Spans exons' TaqMan Gene Expression Assays from Life Technologies. The details of the probes used are shown in Table 2.3.

Table 2.3: Target assays mixes and endogenous control probes used in QPCR. All probes (except EP2) were inventoried and obtained from Applied Biosystems, Cheshire, UK.

Gene symbol	Gene name	Assay ID	Supplier
B actin	Human ACTB(B-Actin) endogenous control	4310881E	Life technologies
PTGS1 (COX-1)	prostaglandin-endoperoxide synthase 1	Hs00377721_m1	Life technologies
PTGS2 (COX-2)	prostaglandin-endoperoxide synthase 2	Hs00153133_m1	Life technologies
IL8	Interleukin 8	Hs00174103_m1	Life technologies
VEGF-A	Vascular endothelial growth factor A	Hs00900055_m1	Life technologies
FLT1 (VGEF-R1)	fms-related tyrosine kinase 1 (vascular endothelial growth factor receptor1)	Hs01052961_m1	Life technologies
VEGF-R2(KDR)	kinase insert domain receptor (vascular endothelial growth factor receptor2)	Hs00911700_m1	Life technologies

Prostaglandin E receptor 2 (EP2) primers and probe for quantitative PCR were designed using the PRIMER express programme (Table 2.4). PE2 primers and probe 1 in 50 dilutions were used for PCR reaction mixture (Table2.5).
For normalisation of quantitative results, B-actin was used as reference gene. 1 µl of undiluted cDNA samples required for each probe with a 1 µl of a1 in 10 dilution of cDNA sample required for B-actin.
Quantification was implemented on an Applied Biosystems 7900 using 96 well plate with a different probe and sample combination in each well.
The thermal cycler conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 second and 60°C for 1 minute. For each probe, samples were run in duplicate and control samples were run for every second plate using DDW and NRT.

Table 2.4: EP receptor2 primers and probe for quantitative PCR from Applied Biosystem

Primers/probe symbol	Primers/probe sequence	Primer/probe Life Technologies Ref. No.
EP2F	5́-GAC CGC TTA CCT GCA GCT GTA C-3́ (forward)	186475051-60-11
EP2R	5-TGA AGT TGC AGG CGA GCA-3́ (reverse)	186475051-70-2.1
EP2PROBE	5́-CCA CCC TGC TGC TGC TTC TCA TTG TCT-3́ (6-carboxyfluoroscein labeled)	186475051-80-1.1

Table 2.5:PCR reaction mixture

Reagents	**EP2 primers/probe added volume (ul)**	**added volume for the other assays (ul)**
2xTaquMan mastermix	**12.5**	**12.5**
20xtarget assay mix / probe for EP2	**1.25**	**1.25**
DDW	**3.75**	**10.25**
EP2F	**3.75**	**-**
EPS	**3.75**	**-**

2.5.5 **Selection of endogenous control gene**

Expression levels of target genes should be normalised to expression levels of internal control genes as normalisation is a common feature of most transcript quantification techniques. The endogenous control gene (housekeeping gene) should show a constant expression level in the tissues of interest. However, it has been demonstrated by a number of studies that frequently used control genes such as ß-actin and GAPDH display significantly variable transcript levels not only in cells of different histological origin but also under various physiological or experimental conditions[196]. This study examined the stability of expression levels of three endogenous control genes ß-actin, glyceraldehhde-3-phosphate dehydrogenase (GAPDH) and 18S in six tissue samples of endometrium, and five myometrium and uterine fibroids tissue samples in order to identify the best one to use as endogenous control gene for normalisation, the expression profile for each gene was determined for these samples. Each taqMan Assay was run in duplicate for each sample. The Ct values were then used to determine the average Ct and Standard Deviation (SD) for each gene across the different samples. The SD was used to identify the gene with the lowest SD (Table2.6). The result shows that B-actin was the control gene with narrowest SD variation among the samples (SD of B-actin was 1.85; SD of GABDH was 2.06 and 3.78 SD of 18S). Although S18 was abundant in all uterine tissue, it was the least stably expressed gene with highest variability amongst the genes (Figure 2. 3) as well as the widest SD variation. Therefore, 18s was excluded as endogenous control gene. To confirm the result ß- actin and GABDH were examined for a further 11 endometrial samples and ß-actin had the narrowest SD .Thus I chose ß-actin as the best endogenous control gene in this study.

Table 2.6:Differences in RNA transcription level between β--actin, GAPDH and 18S in uterine tissue.

	Endometrial samples	Myometrium samples	Fibroids samples
	mean Ct value/SD		
B- actin	24.47/1.85	27.68/3.60	23.96/1.74
GAPDH	26.20/2.06	30.29/4.66	27.48/3.68
18S	17.66/3.78	25.37/6.80	16.46/2.61

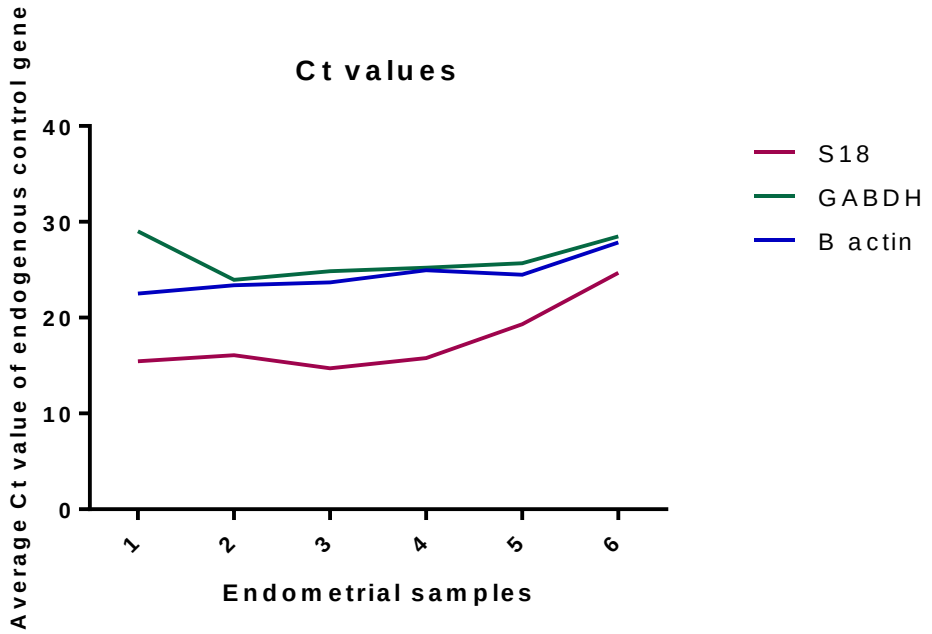


Figure 2.4: Variation of RNA transcription level of β-actin, GAPDH and 18S in endometrial samples in women with HMB.

2.5.6 **Analysis and detection of the target gene expression relative to β-actin**

PCR data were captured and analysed using the automated photometric detector and detection software (ABI Prism 7900, Sequence Detection System 2.3).The threshold value for each probe was determined and used throughout for all PCR cycles using that probe (Table2.7). The threshold value indicates the background level of fluorescence for that probe.

The cycle threshold (Ct) value indicates the cycle number at which the fluorescence passes the set threshold value (background value); therefore it is a way to determine the concentration of the gene of interest. Ct values were then determined for each well and since samples were run in duplicate, the mean Ct value was used and where the difference between Ct values was more than 1.5 Ct values, they were excluded and samples repeated. The expression was then determined relative to the ß-actin value for the sample which provides the Delta Ct (ΔCt) value. ΔCt value is calculating by subtract the Ct value of endogenous control from the Ct value of target gene. The lower the Ct value, the greater the amount of gene present in the sample.

Table 2.7: Threshold values for relevant genes

Gene symbol	Threshold value
ß-actin	0.360
COX-1	0.200
COX-2	0.194
IL8	0.247
VEGF-A	0.230
VEGF-R1	0.264
VEGF-R2	0.200
EP2	0.200

2.6 Immunohistochemistry (IHC)

Standard IHC techniques were performed to localise a number of cellular proteins within the human endometrium, myometrium and fibroid tissue. All protocols were optimised to determine the best condition for maximal immunostaining with minimal background staining.

2.6.1 Antibodies

Antibodies are host proteins found in plasma and extra cellular fluids that serve as the first response and comprise one of the principal effectors of the adaptive immune system. They are produced in response to molecules and organisms, which they ultimately neutralize and/or eliminate. The ability of antibodies to bind an antigen with a high degree of affinity and specificity has led to their abundant use in a variety of scientific and medical disciplines. As a reagent, there is no other material that has contributed directly or indirectly to such a vast range of scientific detections. Their use in diagnostic analyses and as therapeutics has had a profound impact on the improvement of health and welfare in both humans and animals.

Antibodies are glycoproteins secreted by specialized B lymphocytes known as plasma cells. Also referred to as immunoglobulin (Ig), because they contain a common structural domain found in many proteins, antibodies are composed of four polypeptides. Two identical copies of both a heavy and light chain are held together by disulphide and non-covalent bonds, and the resulting molecule is often represented by a schematic Y-shaped molecule (Figure 2.4).

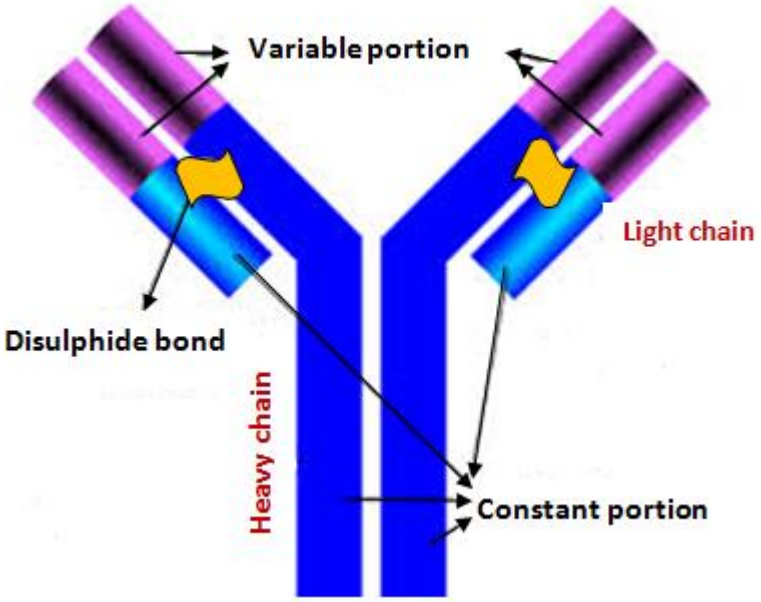


Figure 2.5: Pictorial representation of a typical antibody structure, showing individual chains and regions of variability and constancy.

Depending on the Ig class, up to five structural molecules may be combined to form any one antibody. In mammals, there are five classes of Ig (IgG, IgM, IgA, IgD, and IgE).In select mammals, IgG and IgA are further subdivided into subclasses, referred to as isotypes, due to polymorphisms in the conserved regions of the heavy chain. Ig class determines both the type and the temporal nature of the immune response. All primary antibodies used in this research were the IgG class. Some were polyclonal in nature such as COX-2, IL8, PGE2, VEGF, and others monoclonal in nature such as CD56, CD68, and NE. The variable domain is specific to the target protein and is responsible for antigen binding. Polyclonal antibodies are produced by a variety of cells within an animal species. In that, they are immunochemically dissimilar and may react against various epitopes on the antigens against which they have been raised (Figure 2.6).

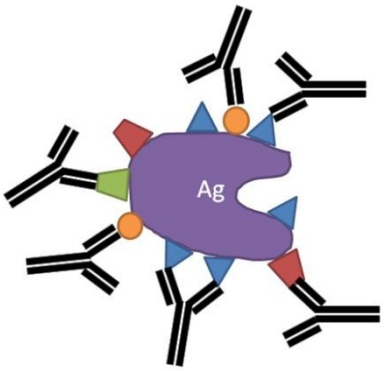


Figure 2.6: polyclonal antibodies rose against an antigen (Ag).

2.6.2 **Formalin-fixed paraffin embedded sample block process**

Tissue processing took place in the pathology laboratory in Glasgow Royal Infirmary and the Southern General Hospital. The usual fixative for paraffin embedded tissues is neutral buffered formalin (NBF). 10% NBF contains 4% formaldehyde by weight and is therefore equivalent to 4% paraformaldehyde. Optimal formalin fixation requires adequate time to form cross links, generally ≥ 24 hours, at room temperature. After this, paraffin processing was performed by taking the cassettes through a series of graded ethanol alcohol (EtOH) baths to dehydrate the tissues and then into xylene. Hot paraffin can then permeate the tissues. Finally, embedded tissue in paraffin block process took place by adding the tissue cassette into melted paraffin according to the process instructions.

2.6.3 **Sections preparation**

Paraffin embedded sample blocks of the study samples were collected from the pathology department archive, Glasgow Royal Infirmary (GRI) and the new pathology department archive in the Southern General Hospital (2012 onwards) after obtaining the histopathology laboratory number. Sections were cut using a microtome (Leica RM 2135) 5µ thick. Because of the difficulty in cutting fibroid sections, these were cut to a thickness of 3 µ and mounted on superfrost glass slides (BDH, Merck House, and Poole) then the Slides heated overnight in oven at 45°C.

2.6.4 **ABC IHC method**

2.6.4.1 Deparaffinising and Rehydration

Xylene was used to remove all paraffin from the tissue sample to give the antibodies complete access to the tissue. Before clearing, the samples were heated to 55°C for 35 minutes to melt the paraffin and then washed multiple times with xylene to remove the paraffin. Next, the xylene was removed by graded washes with xylene and ethanol, and then the sample was rehydrated through graded washes of ethanol in water, ending in a final rinse in 0.01M phosphate buffered saline (PBS). From this point until final mounting, the slides should remain wet to avoid nonspecific antibody binding and high background staining.

2.6.4.2 Antigen Retrieval

Formaldehyde forms methylene bridges between proteins, which can obstruct the epitope (a short amino acid sequence that the antibody is able to recognize as a part of the antigen) recognition by the primary antibodies. Either of two methods was used to remove these bridges which were heat-induced epitope retrieval (HIER) and proteolytic-induced epitope retrieval (PIER). Temperature, pH and time of incubation are critical factors that must be optimised for proper antigen unmasking without causing morphological damage. Sodium citrate (pH 6) and Tris/EDTA (pH 8) buffers were used as appropriate for microwaving sections in pressure cooker.

2.6.4.3 Staining methods

All immunohistochemistry (IHC) performed by using an indirect staining method, where an enzyme labelled secondary antibody directed against the primary antibody (Figure4). This allows for amplification of the primary antibody-antigen complex thereby enhancing the target signal. Furthermore, the use of a biotinylated secondary antibody allows for subsequent incubation with pre-formed avidin-biotin complex thereby the use of the high affinity that avidin has for biotin can lead to further amplification of signal. Because of the high affinity of avidin for biotin the binding can be strongly irreversible. If these complexes

attached to an enzyme-substance system, the amplified antibody-antigen complex can be visualised. This tertiary signal amplification increases the sensitivity of staining techniques, which helps to reduce unwanted background staining.

2.6.4.4 Controls

Validation of all IHC protocol was carried out by the inclusion of control tissue slides. To assure the primary antibodies worked specifically to their target, the inclusion of positive and negative controls were required. For negative controls, the primary antibodies were omitted. For the positive controls, tissue samples, which are known to express the protein of interest, were used in the experiment (Table 2.8). This inclusion is required to ensure that the immunostaining achieved is within the expected cellular compartments.

Table 2.8: The used positive control tissues for different antigens in this study

Antigen	Positive control tissue
VEGF	Kidney
PGE2	ovary
IL8	Tonsil
COX-2	Colon
CD56	Colon
CD68	Tonsil
NE	Tonsil

2.6.4.5 Background immunostaining

Before using antibodies to detect proteins by immunohistochemistry (IHC), all epitopes on the tissue sample were blocked to prevent the nonspecific binding of the antibodies. Otherwise, the antibodies or other detection reagents may bind to any epitopes on the sample. Therefore, the blocking step for IHC was

performed just prior to incubating the sample with the primary antibody. The general protocol was to incubate the fixed, embedded, mounted, cleared and unmasked IHC sample with the appropriate blocking buffer for 30 minutes at room temperature based on the optimised protocol specific to each antibody and target antigen. Normal serum was the common blocking reagent, because the serum carries antibodies that bind to reactive sites and thus prevents the nonspecific binding of the secondary antibodies used in the assay. A critical factor, though, is to use serum from the species that the secondary antibody was generated in, as opposed to the species of the primary antibody. Serum from the primary antibody species would bind to reactive sites, but the secondary antibody would recognize those non-specifically-bound antibodies along with the antibodies bound to the target antigen. Prevention of peroxidase activity and reduction of background staining can be obtained by submerging the section in hydrogen peroxide (H2O2). In addition, incubation of sections in avidin/biotin solution to block avidin /biotin activity can result in very low background staining. Standard IHC techniques were performed to localise a number of cellular proteins within the human tissue. All protocols were optimised to determine the best conditions for maximal immunstaining with minimal background staining.

2.6.5 **General immunohistochemistry protocol**

Tissue sections of 3µ thickness were heated before use in oven at 55°C for 35 minutes. Slides were then dewaxed by placement in xylene for two ten-minute episodes. Afterward, sections were rehydrated through graded alcohols,100% ethanol (2× 5min), 95% ethanol (2× 5min), 90% ethanol (2× 5min)and 70% ethanol (5min)before receiving five-minutes wash in 0.01 M phosphate buffered saline (PBS,PH 7.6). Endogenous peroxidase activity was blocked by exposing slides to 0.5% H_2O_2 in Methanol (300 ml Methanol + 5 ml H_2O_2) for 30 minute after two washes in PBS (2×10min). Antigen retrieval was then carried out by putting buffer solutions in pressure cooker (Lakeland Plastics Ltd, Cumbria, UK) for 15 min at full power to come to the boil, then slides added to the buffer solutions and slides, microwaved for 8min on full power (after 3 min the cooker should be at pressure and slides microwaved under pressure for 5min), followed by a 20 minute cooling period in the same buffer solutions.

Sections were then washed in distilled water for 5 minutes then in PBS for another 5 minutes. After drawing a wax ring around each tissue section, sections were incubated in the appropriate blocking, non-immune serum for 30 minutes in an humidified chamber at room temperature (the blocking serum was for the same species that secondary antibodies were made in) before incubating them overnight at 4°C within the primary antibody solution.

Immunohistochemical staining techniques were optimised for each individual protein of interest. The protocols used for individual primary antibodies are summarized in Tables 8&9. Positive controls were used in every protocol to determine the best condition for maximal immunostaining with minimal background staining. Sections were washed in PBS (2×5min) then incubated for 30 minute in a biotinylated secondary antibody (an antibody with biotin covalently linked to its structure raised against the specific species of the primary antibody) 1 in 200 dilutions.

During this incubation time, a solution of preformed avidin/biotin-peroxidase enzyme complex (ABC Elite, VICTASTAIN, PK6100, and Vector Laboratories kit) was prepared 30 minute before use. Avidin /biotin solution was prepared by adding two drops of A buffer to 5ml PBS and mixed well then adding two drops of B buffer, which are included in Vector Laboratories kit , and mixed well, then incubated for 30 minute in room temperature. The avidin- biotinylated peroxidase complexes are small and highly active, allowing greater tissue diffusion and binding to the biotinylated secondary antibody. The peroxidase-based sensitive detection system is more sensitive than regular ABC methods, allowing better detection of biotinylated targets and hence less detection of non-specific background antigens. Sections were washed in PBS (2×5min) in between steps before applying the next incubation. Peroxidase substrate, diaminobenzidine tetra hydrochloride (DAB, DAKO Corp, Ca, USA) was then employed as the chromagen. The peroxidase enzyme leads to oxidation of the DAB substrate, resulting in a sTable enzyme –substrate complex (reaction time was for 10 min) and a brown precipitation, allowing visualization of the sites of positive immunoreactivity.

Sections were washed for 5 minute in distilled water before counter staining with Harris' Haematoxylin (for 10-15 sec.).Subsequently, sections were washed with tap water for seconds then dehydrated in ascending grades of ethanol and then final incubation in xylene (2× 10 min) was performed prior to mounting in

DPX. The used IHC protocol for each marker was summarised in Table 2.9 &Table 2.10.

Table 2.9: Summary of IHC protocols

Antigen	Primary antibody			Secondary antibody			Antigen retrieval	Tertiary amplificationo	Negative control
	Species	Conc.	incubation	Species	Conc.	Incubatio			
VEGF- A	Goat	1:200	4 °C Over night	Horse - biotinylated	1:200	30 mins. RT	0.01 M - Na citrate	ABC Elite Vectastain	Omit PA
PG E2	Rabbit	1:200	4 °C Over night	Goat- biotinylated	1:200	30 mins. RT	0.01 M- Na citrate	ABC Elite Vectastain	Omit PA
IL 8	Goat	1:50	4 °C Over night	Rabbit- biotinylated	1:200	30 mins. RT	0.01 M- Na citrate	ABC Elite Vectastain	Omit PA
COX-2	Goat	1:500	4 °C Over night	Rabbit- biotinylated	1:200	30 mins. RT	0.01 M- Na citrate	ABC Elite Vectastain	Omit PA

Table 2.10: Summary of IHC protocols for leukocytes

Antigen	Primary antibody			Secondary antibody			Antigen retrieval	Tertiary amplificationo	Negative control
	Species	Conc.	incubation	Species	Conc.	Incubatio			
CD56	Goat	1:200	4 °C Over night	Horse - biotinylated	1:200	30 mins. RT	0.01 M - Na citrate	ABC Elite Vectastain	Omit PA
CD68	Rabbit	1:200	4 °C Over night	Goat- biotinylated	1:200	30 mins. RT	1mM EDTA	ABC Elite Vectastain	Omit PA
NE	Goat	1:50	4 °C Over night	Rabbit- biotinylated	1:200	30 mins. RT	proteinase K	ABC Elite Vectastain	Omit PA

2.6.6 **Scoring and analysis of immune-reactivity**

The immunostaining of intensity of epitopes in all antibodies stained sections was assessed in the semi-quantitative manner on the 4- point scale: 0= no immunostaining, 1= mild immunostaining, 2= moderate immunostaining, 3= intense immunostaining. In endometrial sections, glandular and stromal tissue scored separately. The 90% of tissue sections weremeasured with a computerised image analysis system (Version 4.0, Digital Image Hub, Leica biosystems) and 10% were scored by the research fellow. For COX-2, PGE2, and IL8, double scoring with the same research fellowwas performed. The correlation coefficientbetween manual scoring, automated scoring and between double scoring methods wasaccepted at ≥ 0.7. Detail of scoring method for the used antibodies was provided in each relevant chapter.

2.7 Statistics

Descriptivestatistics were used when it was appropriate. TaqMan data were collected on Microsoft excel sheet. Mann-Whitney test was used to compare between groups for non-parametric data and results expressed in median values (95%Cl). ANOVA and Kruskall-Wallis test used as appropriate. Spearman correlation coefficient used for association between variables. Fisher test was used to compare between categorical data Results were statistically significant with p value ≤ 0.05. Details of when each test used was addressed in each relevant chapter. Minitab 16 Statistical Software was used for data analysis and GraphPad Prism 6 was used for creating the graphs.

Chapter 3

3. Assessment of menstrual blood loss

3.1 Introduction

Heavy menstrual bleeding (traditionally known as menorrhagia) is a common complaint in gynaecology. It not only affects women's quality of life but leads to iron deficiency anaemia [197-200]. Heavy menstrual bleeding is subjectively defined as excessive menstrual blood loss, which interferes with a woman's physical, social, emotional, and/or material quality of life[83]. It can occur alone or in combination with other symptoms. Objective assessment of blood loss defines an upper limit of 80ml during the whole period[201].In fact ,there is a wide variation in the quantity of blood lost by women at menstruation[202, 203]. Clinicians usually enquire about the number of sanitary towels and tampons used, number of days of bleeding ,the presence of clots and flooding episodes, the duration of bleeding and measure the haemoglobin in order to estimate the severity of the complaint and determine treatment. However, there is no correlation between menstrual blood loss and these parameters[204]. Approximately 50% of women presenting with the complaint of heavy menstrual bleeding have an average blood loss<80 ml during the completely period [192, 204]. Therefore, history alone is not reliable indicator of menstrual blood loss [205, 206].

3.1.1 Alkaline Haematin Method

Objective assessment of menstrual blood loss using dedicated and time-consuming methods is not available for routine clinical use. This includes the alkaline-haematin-method which is the current gold standard method for quantification of menstrual blood loss[207]. However, this method is expensive and inconvenient for patients. Nevertheless, it is a very valuable method in scientific research. In this method, as Hallberg and Nilsson descried[207], measurement of menstrual blood loss involves extraction of haem from used sanitary towels using 5% sodium hydroxide, (about 100ml/ sanitary product), then incubation of the sanitary items in the 5% sodium hydroxide solution for 48 hours to allow conversion of haemoglobin to haem. During the same time period a stored sample of the patient's venous blood should be used to create a 1:200 dilution of blood with 5% sodium hydroxide, an aliquot should be stored alongside the menstrual blood collection.

After 48 hours, an aliquot from the mixture of sanitary products and 5% sodium hydroxide is obtained and filtered and diluted by measured addition of further sodium hydroxide, to create a close colour match with the control venous blood solution. From the theoretical total volume of sodium added, is therefore possible to calculate by multiplying the added volume by dilution factor. The optical density (OD) of menstrual blood loss solution and venous blood sample are then measured using spectrophotometry. The menstrual blood loss is then calculated as a quantity of the patient's own venous blood using the following equation[208].

$$\text{MBL}=\frac{(OD of\ Menstrual\ Blood\ Solution\ X\ Total\ Volume\ of\ added\ NaOH)}{(OD\ of\ Venous\ Blood\ x\ 200)}$$

3.1.2 Pictorial Blood Loss Assessment Chart (PBAC)

Pictorial Blood Loss Assessment Chart (PBAC) is a Semi-quantitative assessment of blood loss that compares the sanitary item's stain with given diagrams[192]. This method has been developed as a simple and cheap technique of menstrual blood loss assessment which is more accurate than recording the number of towels and tampons used since ittakes into account the degree to which individual items are soiled with blood. This is an important factor as women use a varying quantity of towels and tampons to collect similar amounts of menstrual blood[203]. Using an appropriate cut-off point would have high positive predictive and negative predictive values regarding heavy menstrual blood loss[203, 209]. Other studies reported that the chart was useful with 88% sensitivity, 52% specificity and a false positive rate of 59% [210]. Nevertheless, reproducibility of results of PBAC was challenged in this study and other studies [203, 209, 211]. In addition, a recent study found that PBAC is a simple and accurate tool for semi-objective measurement of menstrual blood loss that can be used easily in the clinical field [212]. The correlation between pictorial assessment results and values measured by the alkaline-haematin-method was comparatively good, however the amount of blood not captured by sanitary substances is significant[213].

3.1.3 Other methods of blood loss assessment

Other bleeding characteristics such as the number of sanitary materials used is highly dependent on the socio-economic status and the individual hygienic needs [203]. Although simple non-laboratory methods such as, counting and weighing sanitary towels and tampons, and sanitary items or bleeding diaries are widely used, they only provide qualitative information on menstrual blood loss which makes them of less values [213].

3.2 The aim of this chapter

Menstrual blood loss was estimated in the study in order to confirm that participants had heavy menses. Additionally, we attempted to find out a correlation between the heaviness of blood loss and the expression of the relevant vascular markers (VEGFA, PGE2, IL8, COX-2, and COX-1) in the endometrium.

3.3 Material and method

3.3.1 Subjects

All target subjects in this study were women who were experiencing regular heavy menstrual bleeding with cyclic interval between 21 and 35 days. Pictorial Blood Loss Assessment Chart (PBACs) was provided to 79 women who had their uterine biopsies taken with their consent to participate in the study.

3.3.1.1 Group 1

54 women were studied in the first group (women were underwent hysteroscopy as part of their investigations for heavy menstrual bleeding).

3.3.1.2 Group 2

16 women were in the second group (women had hysterectomy as treatment for their uterine fibroids).

3.3.1.3 Group 3

Nine subjects in were women had uterine artery embolisation as treatment for their uterine fibroids.

3.3.2 Pictorial Blood Loss Assessment Chart (PBAC)

PBAC was validated by Higham in 1990. The chart consisted of a series of diagrams representing slightly, moderately, and heavily soiled tampons or towels, a mark or number was made in the appropriate box at the time that each towel or tampon was discarded. Women with a pictorial chart score of 100 or more (cut-off point) during one menstrual period, which corresponds to a blood loss of more than 80 ml considered the upper limit of normal for menstrual blood loss and thus being heavy menstrual bleeding.

3.3.2.1 Distribution of PBAC

The women included in this study had an explanation of how PBAC should be used and a simple instruction about how to fill in the chart when they attended the hysteroscopy or the radiology outpatient's clinic (the first and the third subject group respectively) and all subjects were asked for completion with their next menses. Women who had a hysterectomy for their fibroids were provided with PBAC at the pre-assessmentclinic or by post to their address a few weeks before their surgery according to the waiting list. In an attempt to avoid the effect of prescribed drugs for the HMB, participants who attended the hysteroscopy clinic were asked to complete the PBAC when they had the next period, and they were provided with a stamped envelope to return it back to the research office address (3rd floor McGregor building, Glasgow Western Infirmary). Subjects in the second and the third group were asked to bring the PBAC when they come to have either their hysterectomy or uterine artery embolisation performed. A copy of a PBAC chart, completed by a subject in the study, is provided in appendix 2.

3.3.2.2 Calculation of PBAC

A slightly soiled towel was scored 1 point, a moderately soiled towel was scored 5 points, and a towel that is saturated with blood was scored 20 points.
A slightly stained tampon was scored 1 point, a moderately stained tampon was scores 5 points and a tampon that is fully saturated was score 10 points. Blood loss in clots and flooding were also indicated and calculated as follows. A clot size of 1p scored 1 point; a 50p-sized clot scored 5 points, and flooding also scored 5 points. The total score was calculated by multiplying the number of used towels by the relevant score point. A score of 100 or greater was the cut-off point indicates heavy menstrual bleeding.

3.4 Statistics

PBAC score was not normally distributed so correlations were tested with the Spearman rank test for correlation between observations. Differences between groups were evaluated by Mann-Whitney test and Kruskall-Wallis tests, as appropriate.

3.5 Results

In the hysteroscopy clinic, about 25% of subjects (13 participants) did not return their Pictorial Blood Loss Assessment Chart (PBAC), three of these women were women with uterine fibroids and10 of them had no uterine pathologies. Therefore, they were excluded from our analysis. In the second subject group, all PBACs were completed and returned to us. The PBAC score of 66 women who complained of heavy menstrual bleeding ranged from 8 to 1860 (median 343.5). Nearly 11% of women who had no hormonal therapy (8 subjects) had PBAC score less than 100 (range between 8 and 99, median 54.5). Three women out of seven who were on a continuous progestogen had PBAC score less than 100 (two of them in the first group and had score of 11 &12 and the third subject in the second group had score of 36).

The relationship between the pictorial chart score and the length of bleeding time is shown in Figure3.2. This plot shows that women had an average of 7 days of bleeding time every month, and there was positive correlation between the PBAC score and the longer duration of the bleeding time (r=0.397,P= 0.001, Spearman correlation).

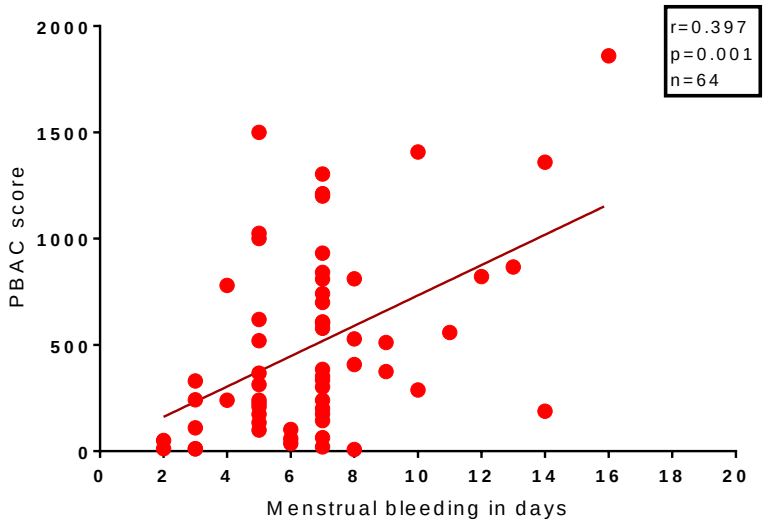


Figure 3.1: Scatterplot illustrating PBAC score against days of menstrual bleeding (length of period).

The median of the pictorial chart score for women with uterine fibroids and women with no uterine pathologies, whether they had pictorial chart score more or less than 100 is recorded in Table3.1.

Table 3.1: Summary of the median of PBAC score for participants in the study

PBAC score	Total subjects number	Median of PBAC score	No pathology	Fibroid	Mann-Whitney	
			No./score median	No./score median	95%Cl	P value
≥ 100	57	515.5	18/674	38/464	-1999.9,382.8	0.735
< 100	7	54.5	1/11	6/61	5.9,91.0	o.066

Mann-Whitney test was used to find out differences in heaviness of menstrual bleeding between women with uterine fibroids and women with no uterine pathology. There was no difference between the medians of PBAC score for women with no uterine pathology and women with uterine fibroids (median= 515.5, 95%Cl (-199.9, 382.8) & P=0.7). When women with HMB and a score less than 100 were compared, the difference between medians of PBAC score for women with normal uteri and women with uterine fibroids was large (50.5), although it was not statistically significant (95%Cl (5.99-91.00) & p=0.066).

The number of towels and tampons used is plotted against the pictorial chart score for each patient (Figure3.2) to point out the relation between them. Higham in 1990 found a positive correlation between the total number of sanitary items used and the menstrual blood loss in ml (r=0.74).

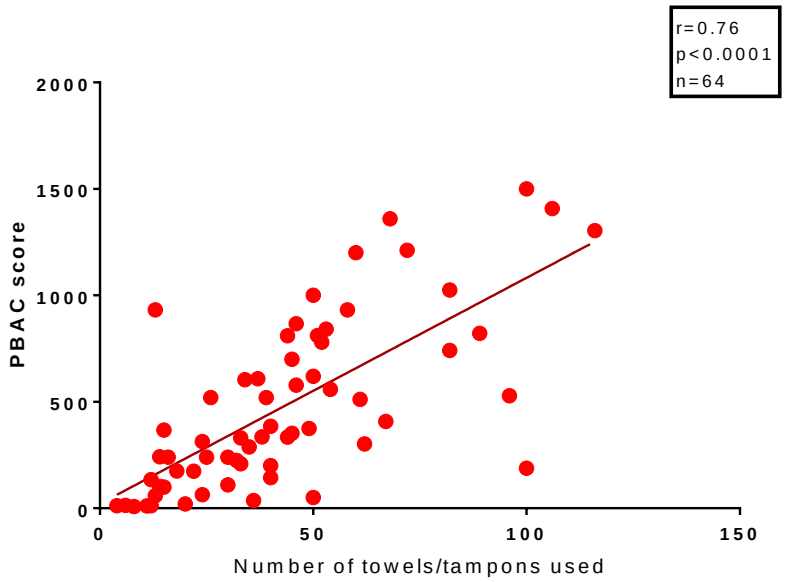


Figure 3.2: Scatter plot illustrating the number of sanitary towels and tampons used against PBAC score. Statistical analysis was performed using a Spearman correlation test.

The finding in this study estimated the correlation between number of towels and tampons used and the pictorial scores, and a positive correlation was found ($r= 0.76$, $P<0.0001$, $n=65$).

The genes of interest in this study have been recognised as those involved in the control of menstrual bleeding and pictorial chart has been approved as a consistent blood loss assessment technique, thus there may be a relation between the level of each gene expression and PBAC score. However, our data could not find a relationship between these variables and the PBAC score. Furthermore, there was no difference between the endometrium expression of cyclooxygenase1, cyclooxygenase2, interleukin 8 and prostaglandin E_2receptor2 (EP2) genes in patients with PBAC score less than 100 and patients with PBAC score ≥ 100 (Figure 3.3-3.5). However, VEGFA showed higher expression in endometrium of women with low PBAC score than the expression in endometrium of women with high PBAC score (95% Cl (68.1, 638.8) & P= 0.01). All these women had no hormonal therapy.

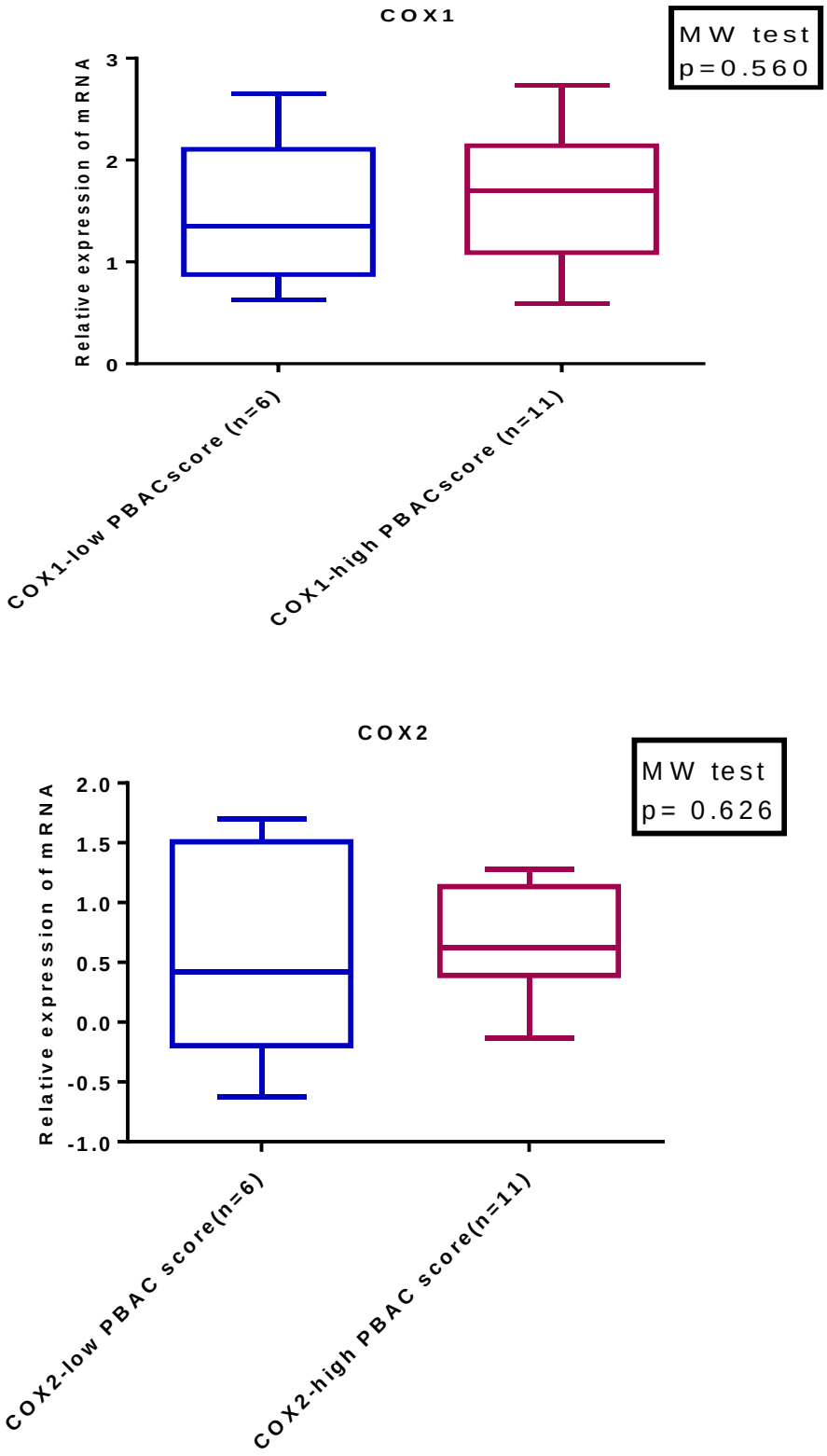


Figure 3.3: The box plot in this Figure illustrates the difference in the relative expression of COX-2 and COX-1 mRNA in endometrium between women with low PBAC score and others with high PBAC score. (Log scale is depicted).

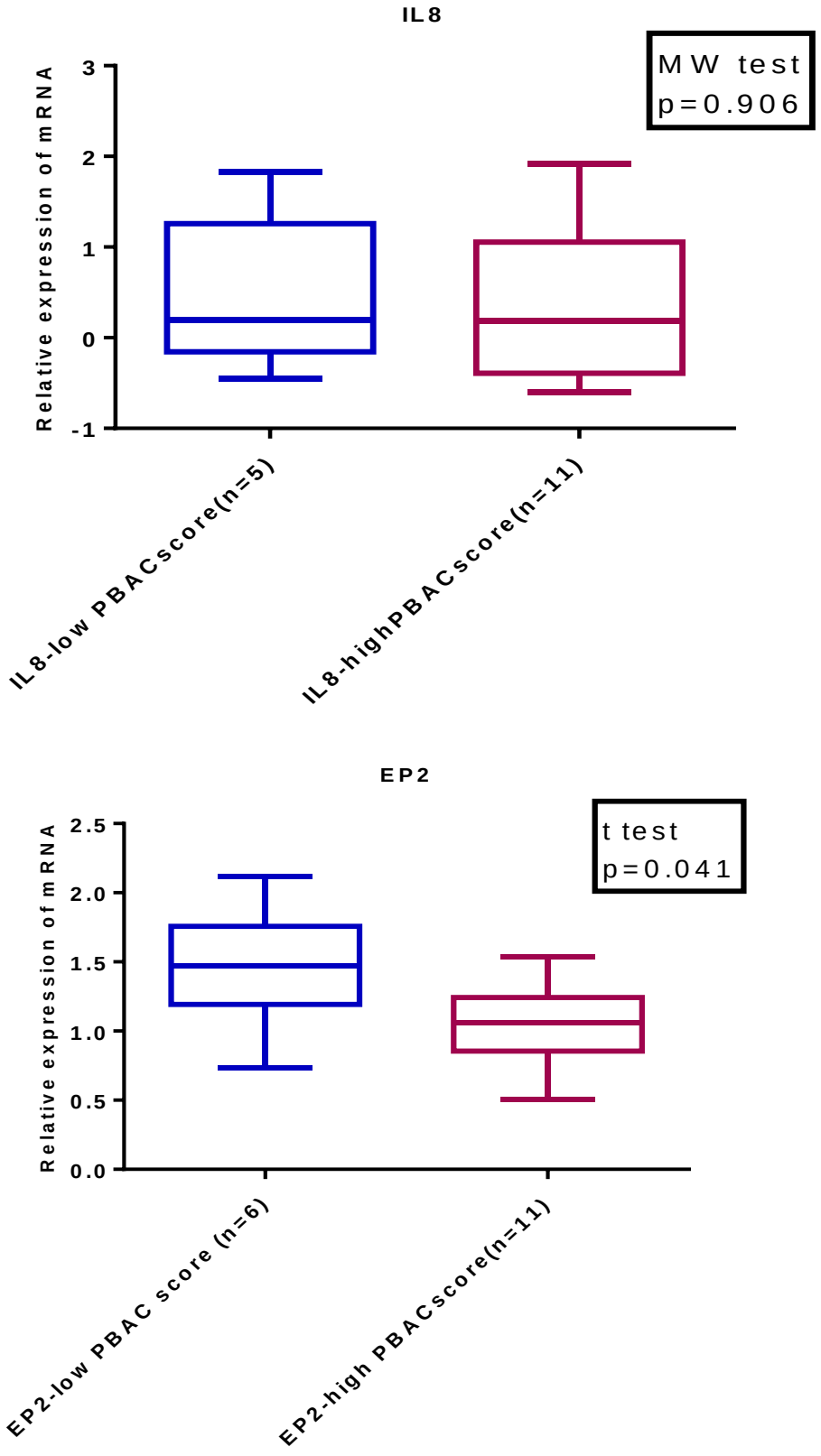


Figure 3.4: The box plot in this Figure illustrates the difference in the relative expression of IL8 and EP2 mRNA in endometrium between women with low PBAC score and others with high PBAC score.(Log scale is depicted).

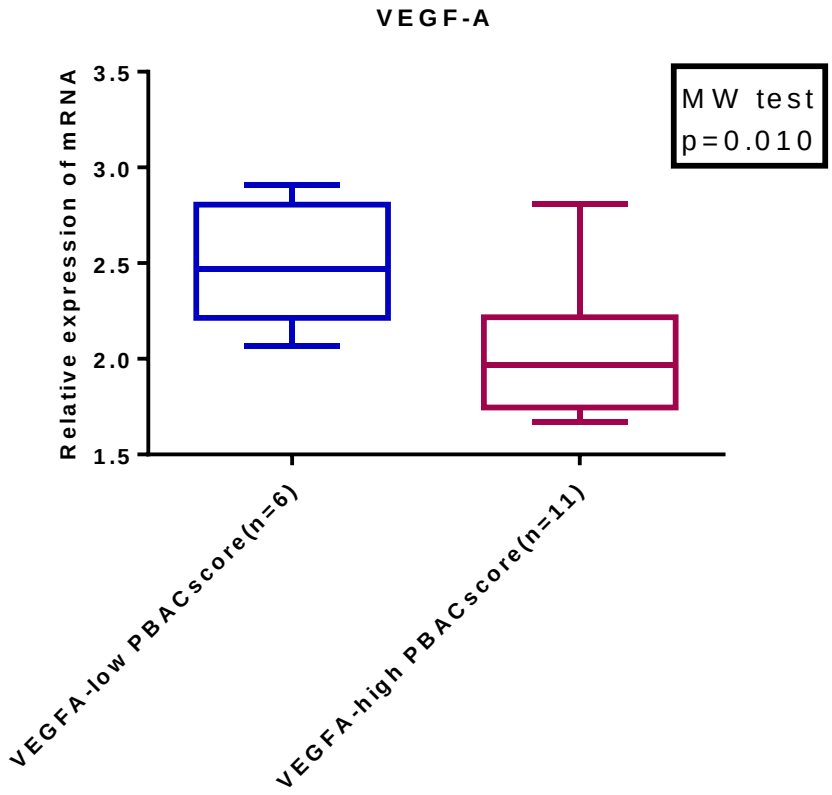


Figure 3.5:The box plot in this Figure illustrates the difference in the relative expression of VEGF-A mRNA in endometrium between women with low PBAC score and others with high PBAC score.(Log scale is depicted).

3.1 Discussion

The pictorial blood loss assessment chart has been approved as a reliable subjective tool for estimating menstrual blood loss that is validated to the objective estimation method of blood loss (alkaline haematin method). In this study, instructions on how to fill in the pictorial blood loss assessment chart were not communicated appropriately to the participants since details of interpretationof the scoring were also included in error. In addition, the description of how to complete the chart which is provided at the bottom of the chart may not have been sufficient for the majority of them to understand exactly how to complete it and it would have been better to show them how to fill in the chart in person. In addition, as already mentioned, the instructions for analysis were accidentally included on the PBAC chart as shown in appendix 2.Women, who were sent the chart by post, had an opportunity to clarify how to complete the PBAC chart during a phone call. Two patients, one in the first group and other in the third group, put only a mark in the appropriate box but they did not indicate the towel number that they had used. One of them (a woman in the third group) had time to provide another completed chart before the performance of her procedure and the other was not included in the result. Although some authors pointed out that about 50% of women who complain heavy menstrual bleeding actually have a normal menstrual blood loss, in this study (excluding women who were on hormonal therapy) only seven women who had complained of heavy menstrual bleeding (10% of participants) had low pictorial chart score (below 100 the usual cut of point). Two of them had pictorial chart score of 97 and 99. It is not clear whether these two scores were due to the patient having normal menstrual bleeding (an average amount of menstrual blood loss <80ml) or due to the monthly menstrual bleeding variation. One of the women with low pictorial chart score, who had a hysterectomy as treatment for her uterine fibroids, had a history of gonadotrphin releasing hormone agonist administration and the patient had the last injection three months before her biopsy was taken. Therefore, the patient was fitted our inclusion criteria. Another woman in the same group admitted that dysmenorrhoea was bothering her rather than the heavy bleeding. One of the

two women in the third group with low pictorial chart score underwent hysteroscopy investigation (under general anaesthesia) a few months before the uterine artery embolisation and the patient thought that the hystroscopic procedure reduced her menstrual bleeding.The remainder of the women with low pictorial chart score had been referred for the first time by their general practitioner to the gynaecologist (hysteroscopy clinic) to investigate their complaint of heavy menstrual bleeding without any previous history of any treatment.

There is aconcern about the accuracy of the way in which some women completed the pictorial chart, particularly since the instructions given contained the methods of analysis which might have influenced the way the charts were completed as described above. The patients were attending the hospital for clinical reasons, and the pictorial blood assessment chart may be considered as an interesting tool but it is not normally part of their clinical evaluation. Hence, some of them may not complete the chart in an appropriate way. Higham and her colleagues in 1990 admitted that they increased the accuracy of the pictorial chart assessment in their study by asking the women to use the uniform types of towels and tampons that were supplied by the trial, while in this study where participants used their own towels and tampons.

The median for pictorial chart scores of 343.5 was high, where it was three fold of the median score for Hingham's population in 1990. Some of observations of pictorial scores in this study were ≥ 1000, which corresponds to approximately a blood loss of ≥750ml[192] (cited in [214]). This very large amount of blood loss would lead to severe anaemia, which was not the case with those patients. These women may use small size and /or low absorbency sanitary materials. Patient's hygiene and perception of the degree of soiling tampons and towels could also be an explanation for these high pictorial score.

The pictorial scores analysis found there was not any difference in the scores between women who complained of heavy menses with uterine fibroids and women with normal uteri, and there was no correlation between pictorial scores and expressions of the genes known to be associated with the control of menstrual bleeding with the exception of VGEFA where expression in the endometrium for women with pictorial scores <100 was higher than VGEFA expression in the endometrium for women with pictorial score ≥ 100. Women who complained of heavy menses and some of whom underwent medical and

surgical intervention for this reason, may have menstrual blood loss within the normal range as pictorial score less than 100 which corresponds to <80ml blood volume, and with higher numbers indicating heavier bleeding. The results of pictorial blood loss assessment in the study are supported by the significant differences in VGEFA mRNA levels between women with heavy menstrual bleeding (≥ 100) and women with normal menstrual bleeding (<100) where this conclusion about the VEGFA levels in normally and heavily menstruated women was previously demonstrated [94]. In this study the levels of mRNA encoding VEGF-A in the menstruated endometrium from women with heavy menstrual bleeding was significantly reduced, the median ΔCt being –7.87 in women with normal menstrual bleeding (range –13.17 to –6.70) and –7.31 in the HMB group with a $P < 0.016$. Nevertheless, other investigations have found an increase in the VEGF- A and it's two receptors in endometrial blood vessels collected from women with heavy menstrual bleeding[215]. This study documents a reduction in the expression of VEGF in endometrium in association with a reduction in menstrual blood loss in women who had heavy menstrual bleeding with uterine fibroids but who were using progestin in the form of a levonorgestrel-releasing intrauterine device[216], meaning that the data are not comparable.

3.6 Conclusion

Pictorial blood loss assessment chart (PBAC) is a semi-objective means of blood loss estimation in the research field with good acceptance by patients.Evaluating the effectiveness of available treatments of heavy menstrual bleeding, such as medical and the less invasive interventional methods, for example, uterine artery embolisation, can be one of the uses of the pictorial blood assessment chart. Unfortunately, for the reasons described above, this part of the research project was unsuccessful and results difficult to interpret. Any conclusions from these data must be interpreted with caution. However, it appears likely that the VEGF-A was less in women with significant HMB as this is in agreement with other studies.

Chapter 4

4. The immune response of human endometrium in presence of uterine fibroids .Is HMB one of consequences?

4.1 Introduction

The human endometrium is an important site of innate immune defence, giving protection against uterine infection. Such protection is vital to successful implantation and pregnancy. Therefore, the human endometrium works as other mucosal surfaces and it produces a wide range of natural antimicrobial peptides, these peptides are expressed by both epithelial cells and endometrial leukocytes and they appear to be governed by the stage of the menstrual cycle[217], and provide protection throughout the menstrual cycle and in pregnancy. There is a strong suggestion that interaction between steroid hormones, leukocytes and locally produced cytokines and growth factors is involved in this protection mechanism [82, 218]. The cellular composition of endometrium is always changing in parallel with the daily cyclic hormonal alternation. After the menstruation and shedding of epithelial tissue, re-epithelialisation is initiated from the stem cells in the glands present in the basalis layers and from the residual raft of luminal epithelium. The full thickness of endometrium is restored by the end of the proliferative phase of the cycle where throughout that phase both stromal fibroblast and cells in association with the blood vessels (endothelial and vascular smooth muscle cells) undergo mitosis. Differentiation of many of these cells including decidualisation of some stromal cells takes place during the secretory phase.

4.1.1 Leukocytes expression in human endometrium

The population of lymphomyloid cells is governed with the different phases of the cycle, thus their total and relative number vary throughout the menstrual cycle and reach the greatest abundance during the premenstrual phase[219]. They include eosinophils, macrophages, neutrophils, endometrial granular lymphocytes (natural killer (NK) cells), T cells, B cells and mast cells. All these cells can synthesise and release an abundance of cytokines and proteases, including the matrix metalloproteinases (MMPs). Subpopulations of these cell types with different phenotypes are present in the endometrium and others with

apparently similar phenotype to those in the endometrium can be functionally different from those in the blood of the same individual. Thus at least some of the cells which traffic into the endometrium must change in phenotype in response to the new environment. In addition, it is not clear whether the increase in the number of leukocyte subpopulations in the late secretory phase is related to in situ proliferation or to migration from the peripheral circulation or both of these processes. Whether these processes are driven directly by ovarian hormones or are mediated by other factors such as chemokines or adhesion molecules is obscure. Leucocytes account for 10% of endometrial stromal cells in normal proliferative and early secretory phase endometrium and numbers increase to over 20% of stromal cells in the mid and late secretory phase[220]. Leukocytes can be found in endometrium within the stratum basalis, where they often form lymphoid aggregates. In the stratum functionalis, they may be scattered singly, form small aggregates around glands and blood vessels or are found in an intraepithelial position as well as within glandular lumina [221]. They are believed to have a role in implantation, decidualization and placentation [222]. The presence of these cells in endometrium is dependent on progesterone although this may be an indirect action[223].

4.1.1.1 Macrophages

Macrophage cells in tissue arise by differentiation of monocytes that migrate from blood. Macrophages display heterogeneity in their phenotype, and subtypes have been described according to expression of MMP-9[30], activin β [224],and MT1-MMP [225].Infiltration of macrophages increases from the proliferative phase throughout the menstrual cycle to reach highest expression in the late secretory phase[220, 226, 227],around the time of implantation and throughout pregnancy[228] and form 20% of the leukocyte population at this time. An increase in the macrophage infiltration in progestin exposed endometrium and abnormal uterine bleeding has been documented[229]. The concentration of macrophages in endometrium is mainly increased around the endometrial glands[230] and at the implantation site in early pregnancy[231]. Macrophages have a role in debris removal at the materno–fetal interface and may help prevent the initiation of an immune response to the trophoblast [232].In addition, the act of phagocytosis of apoptotic cells suppresses the release of

proinflammatory mediators from macrophage cells [233]. In this way, they might act to optimise the inflammatory environment at implantation. Furthermore, macrophages have the ability to actively initiate apoptosis of unwanted cells during tissue remodelling [234] and may play a role in blastocyst invasion. The endometrial macrophage has also been shown to express oestrogen receptor-ß and oestrogen-related receptor-ß [235]. However, the endometrial macrophages do not express the progesterone receptor and oestrogen receptor α [236, 237], but are recruited by a host of macrophage attracting chemokines. Macrophage activation is reflected in a continuous spectrum of phenotypic changes, that rapidly leads to alternation in macrophage function in response to local environmental signals such as surface receptors and secreted molecules[238, 239].These can produce molecules that stimulate and regulate MMP9 such as cytokines[240],tumour necrotising factor α (TNFα) and interleukin-1ß (IL-1ß)[241].

4.1.1.2 Neutrophils

Neutrophils are polymorphonuclear cells and they are the most abundant leukocytes in the human immune system. They contain specific secretory granules which contain a varied range of regulatory molecules. However, these molecules differ according to the neutrophil maturation stage. They initially interact with the wall of the blood vessel during transmigration and subsequently with the endometrial decidua and extracellular matrix (ECM) during passage through the tissue[242]. It is thought that neutrophilsare dominant in the initial leukocyte influx into the tissue[26], and are associated with tissue damage in inflammatory disorders. Neutrophils are identified in endometrium by their morphology and by immunolocalisation of the neutrophil-specific protease-elastase. In normal endometrium the neutrophils are hardly detected, however, there is an abrupt increase in their infiltration in the premenstrual phase[25] and high expression in areas of endometrial breakdown in patients treated with high doses of progestin[230, 243]. Neutrophils as macrophages, have varied phenotypes but only some of them are positive to MMP9[243] or activin ß,which play a role in cells differentiation and apoptosis,[224]. Moreover, some neutrophils have a membrane type MT-MMP,which has a role in activation of latent MMP-2 [29]. Neutrophil

granuleenzymes can activate latent forms of proinflammatory molecules such as interferon gamma (INF-γ) and interleukin-1 β (IL1β)[27, 244]. In addition, intraepithelial neutrophils contain INF-γ which has a role in macrophage activation and this may demonstrate a potential interaction between leukocytes at menstruation[245]. It was pointed out that neutrophils enter the endometrium from the spiral arterioles, since decidualization and the accompanying chemokine secretion occurs initially close to these vessels. The vessels themselves express chemokines at this time [27].

4.1.1.3 Mast cells

The mast cells secrete many vasoactive and pro-inflammatory molecules and share in the inflammatory process by vasodilatation and enhancing the infiltration of leukocytes and thus causing direct tissue damage by releasing their proteases. There are subtypes of mast cells in the endometrium depending on the expression of cell –specific serine proteinases or tryptase, which are present mainly in endometrial functionalis layer. The functionalis is only positive for mast cells with this specific proteinase, chymase is present only in the endometrial basalis layer[32]. Mast cells precursors in peripheral blood reach the uterus and differentiate to the mature form in situe [246]in response to chemokines [247], under the control of steroid hormones [248]. Mast cells activation occurs in response to tissue oedema prior to menstruation[32]. The action of mast cell on extracellular degradation is mainly through activation of MMP production[31],including MMP2 [249]and MMP9[250]as well as collagenase[251]. Some authors point out that mast cells not only activate MMPs but also can produce MMPs themselves, and are stimulated by tumour necrotising factor-α(TNFα) and transforming growth factor-β (TGFβ)[31, 252]. The endometrial stimuli for activation of mast cells with its associated release of mediators are not known. However, endothelins, which are abundant in menstrual endometrium, can activate mast cells via their receptors, providing one potential mechanism. Endothelins themselves are inactivated by mast cell carboxy-peptidase A3 and clearly a balance must be achieved [253].

4.1.1.4 Uterine natural killer cells (uNK)

These cells known as endometrial granular lymphocytes[254, 255], express the natural killer cell antigen CD56[256]. The uNK cells are localised by staining with Phloxina-Tartrazine and by immunolocalisation. They infiltrate into both endometrial stroma and intraepitheial cells but it is not known if there is a difference in function between the cells in the two different sites [257]. Their expression is mainly close to the glands and spiral arteries[258], which suggests a role in vascular remodelling[259].The uNK cells found throughout the menstrual cycle , count for about 15% of the total number of stromal endometrial cells [25], and increases to 25% during the perimenstrual time[254]. This high expression at the late secretory phase may be due toproliferation in situe. The uNK cells are the most prevalent leukocytes at the time of implantation and comprise 70-80% of endometrial leukocytes [22, 260, 261], this increase being enhanced by human chorionic gonadotrphin hormone (HCG)[262]. Furthermore, high uNK infiltration during the secretory phase and in early pregnancy suggests a potential regulation by progesterone, although there was no evidence of nuclear progesterone receptors [263, 264]. In comparison to NK cells in peripheral blood , the uNK cells have cytotoxic activity [265]and have bright CD56 expression [260]. The cytotoxic activity increases from the end of proliferative phase toward the perimenstraul time indicating a general mucosal defence and anti-infection protection mechanism. These cells may be involved in immunomodulation at the time of implantation due to the expression of proteins with immunomodulation function [266]. Although, uNK cells undergo morphological nuclear changes in the premenstrual phase, their high expression at this time indicates their terminal differentiation and imminent non-apoptosis [267, 268]. A range of cytokines are secreted by uNK cells, which have both an immunosuppressive and growth promoting role, and this role may be of greater importance at the end of the cycle in preparation for pregnancy rather than a role in the process of menstruation itself. this concept has been supported by Critchely el at.1999[269].

4.1.2 **Leukocytes and menstruation**

The inflammatory mechanism of menstruation includes complex sequences of events, which depend on interaction between sex steroids hormones and the immune system[82].The fall in progesterone and oestrogen levels in the late secretory phase initiate this mechanism. Progesterone withdrawal is thought to cause inflammatory gene induction, thus leading to an influx of leukocytes. Interactions between these cells and decidualised stromal cells facilitate release of many pro-inflammatory mediators including chemokines, cytokines and prostaglandins. These pro-inflammatory factors activate the leukocyte and boost them to produce and activate a cascade of degradative enzymes, particularly matrix metalloproteinases. Thus, a rapid breakdown of the extracellular matrix, which is supporting the tissue, takes place. Other opinion suggests that sex steroid hormones might simulate the leukocytes directly as many of the CD68 positive cells in peripheral blood express progesterone receptors[270] and also T helper cells in the endometrial basalis express oestrogen receptors[271]. Other studies concluded that large numbers of progesterone receptors are not found in endometrial leukocytes, and steroid hormones most probably influence endometrial leucocyte populations indirectly via products of endometrial stromal or epithelial cells that express steroid hormone receptors[82, 265]. Because of the leukocyte invasion into the endometrium and subsequent production of inflammatory mediators, menstruation has been described as an inflammatory event [272, 273]. Although many of the molecular and cellular changes at menstruation are well known [274, 275], the precise timing of these changes and the interactions that lead to the tissue breakdown and repair are still not fully understood because of a tissue sample taken from a woman during menstruation is likely to contain a combination of pre-menstrual tissue that has not yet started to degrade, tissue that is actively degrading and areas that are actively repairing. These are clearly obvious in histological samples.

4.1.3 **Leukocytes and heavy menstrual bleeding**

Menstruation is characterised by an influx of leukocytes, extracellular matrix modification and increased vascular permeability. Regulation of the initiation, intensity and resolution of the inflammatory response is important for effective implantation and efficient repair after menstruation. As discussed above, inflammation is a key event during menstruation, thus it is reasonable that abnormalities in this process may contribute to menstrual disorders. The cyclical endometrial breakdown and repair are highly regulated by synchronisation between sex hormone production from the ovary, production and activation of pro-inflammatory mediators, and leukocyte influx. Deviation from this co-ordination sequence can lead to menstrual disorders, such as heavy menstrual bleeding (HMB). Increased levels of total prostaglandins in women with heavy menstrual bleeding[108] and the pro-inflammatory cytokine TNFα in the menstrual effluent in these group of women, [94] is a further indication of an exaggerated inflammatory reaction in menstrual disorders.

4.1.4 **Leukocytes and uterine fibroids**

The presence of uterine fibroids, especially submucosalal and intramural fibroids may cause deterioration in the inflammatory process in the endometrium by increasing the pro-inflammatory mediators such as chemokines, cytokines, prostaglandins and matrix metalloproteinases (MMP). The cytokine, transforming growth factor-beta (TGF-β) plays an important role in enhancing the trophoblast cells attachment to the endometrium. This factor plays a role in the proliferation, differentiation and extracellular matrix production, and it is found that it has high expression level in fibroid tissue as well[145]. Thus, it may have a role in uterine fibroid formation as a consequence of abnormal tissue repair and an altered extracellular matrix. The use of gonadotrophin – releasing hormone agonists can suppress the level of TGF-β and its receptor[276],as well as reducing the size of uterine fibroids. Chemokines, which is correspondence to macrophage, may lead to amplification of the inflammatory process in the endometrium overlying the uterine fibroids. These pro-inflammatory mediators increase the leukocyte infiltration into the endometrium mainly by macrophages. Leukocyte mobilisation, with changes in their phenotypes and

activation, provide further degradative enzymes and MMP activators. As a result, further tissue breakdown, and longer bleeding time may occur and affectthe menstrual blood loss. An increased accumulation of macrophages in the endometrium was noted in women with submucosal and intramural uterine fibroid. It is thought that this accumulation corresponds to the high production of monocyte chemoattractant protein-1(MCP-1), the chemokine CCL2, and levels of PGF2α which are also significantly increased in those women[277]. As a result of all this, amplification of the inflammatory reaction in endometrium takes place and a subsequent detrimental effect on the menstrual cycle may result.

This chapter aimed to estimate the infiltration of neutrophils, macrophages and uterine natural killer cells in the endometrium for women with heavy menstrual bleeding and uterine fibroids and compare it to that for women with heavy menstrual bleeding and no uterine pathology.

4.2 Patients and methods

Discussed in materials and methods chapters

4.3 Results

4.3.1 Expression of Neutrophil elastase protein (Neutophils)

The endometrial stromal neutrophil elastase was low in the proliferative, early secretory and mid-secretory phases in each group and markedly increased in late-secretory and menstrual phase. It was significantly different among the phases ($P<0.0001$, ANOVA test) (Figure 4.1). There was no expression of neutrophil elastase in surface and glandular epithelial cells (Figure 4.2). In contrast, there was no significant difference among the women with uterine fibroids and women with normal uteri across the menstrual cycle.

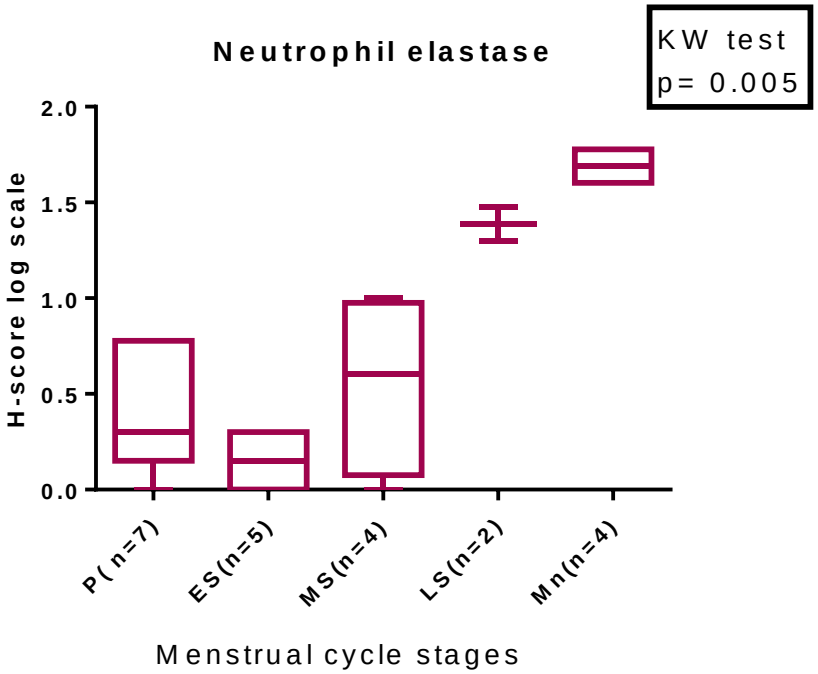


Figure 4.1: The immunostaining score of neutrophils elastase antibody in endometrial stromal cells in women with heavy menstrual bleeding across the menstrual cycle, P (proliferative), ES (early secretory), MS (midsecretory), LS (late secretory), and Mn (menstrual) phases. (Note that y is the loge scale of scoring, t test was used). (Log scale is depicted).

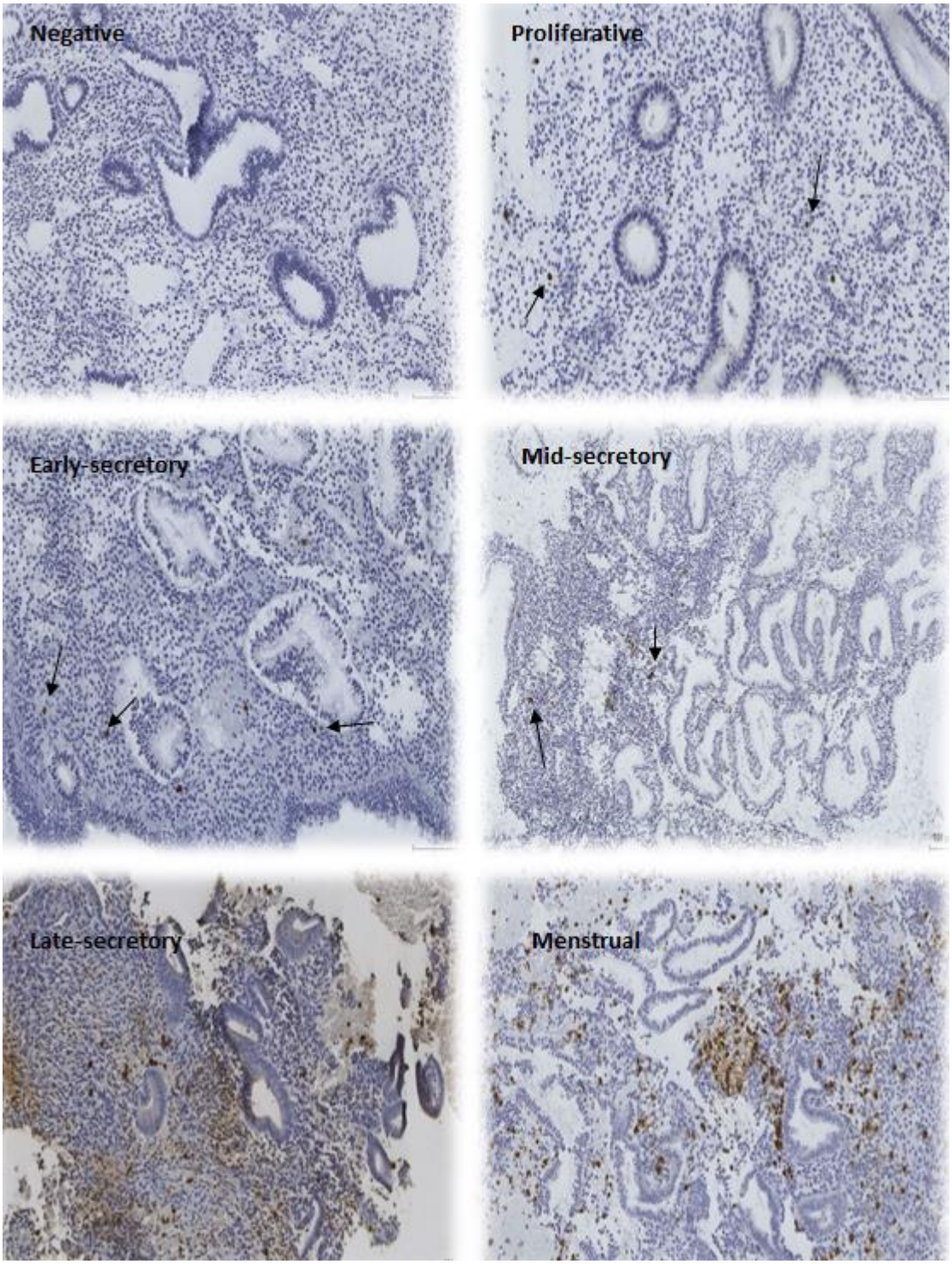


Figure 4.2: Immunolocalization of neutrophils elastase (a specific marker for neutrophils) in endometrial biopsies obtained from women with heavy menstrual bleeding. Immuno-positive stromal cells in brown colour (cells in blue colour are negative for neutrophils elastase) were few during proliferative, early secretory and mid-secretory phases. The immunostaining of neutrophil elastase is upregulated in late-secretory and menstrual phases.

4.3.2 **CD56 +cells (uterine natural killer)**

Immunohistochemistry immunostaing identified CD56 expression across the menstrual cycle in endometrium for both women with idiopathic heavy menstrual bleeding and with uterine fibroids. The expression of stromal CD56 was upregulated across the menstrual cycle in both groups, with low expression in proliferative and early secretory phases and markedly increased during mid-late secretory and menstrual phase. The expression of CD56+ cells was significantly different between mid-secretory and proliferative phase ($P<0.01$) and mid-secretory and early secretory phase ($p<0.05$). On the other hand, there was no difference between other phases (mid-late secretory and menstrual) (Figure 4.3). The infiltration of CD56 was in endometrial stroma mainly around the glands and blood vessels and occurred singly or in aggregates, however, some infiltration of CD56+ cells was noticed in-between epithelial cells (Figure 4.4).

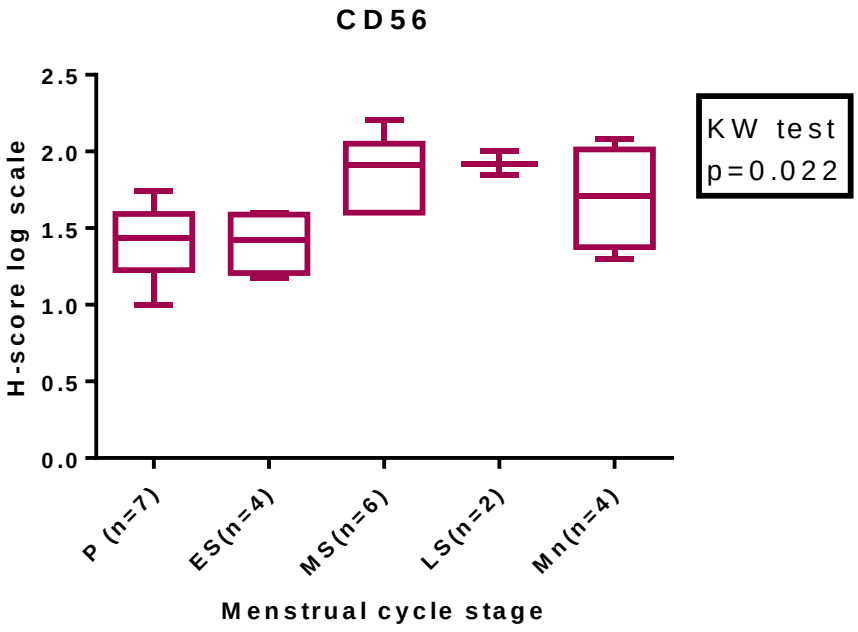


Figure 4.3: Immunostaining score of CD56 in endometrial stromal cells across the menstrual cycle, P (proliferative), ES (early secretory), MS (midsecretory), LS (late secretory), and Mn (menstrual) phases. (Log scale is depicted). .

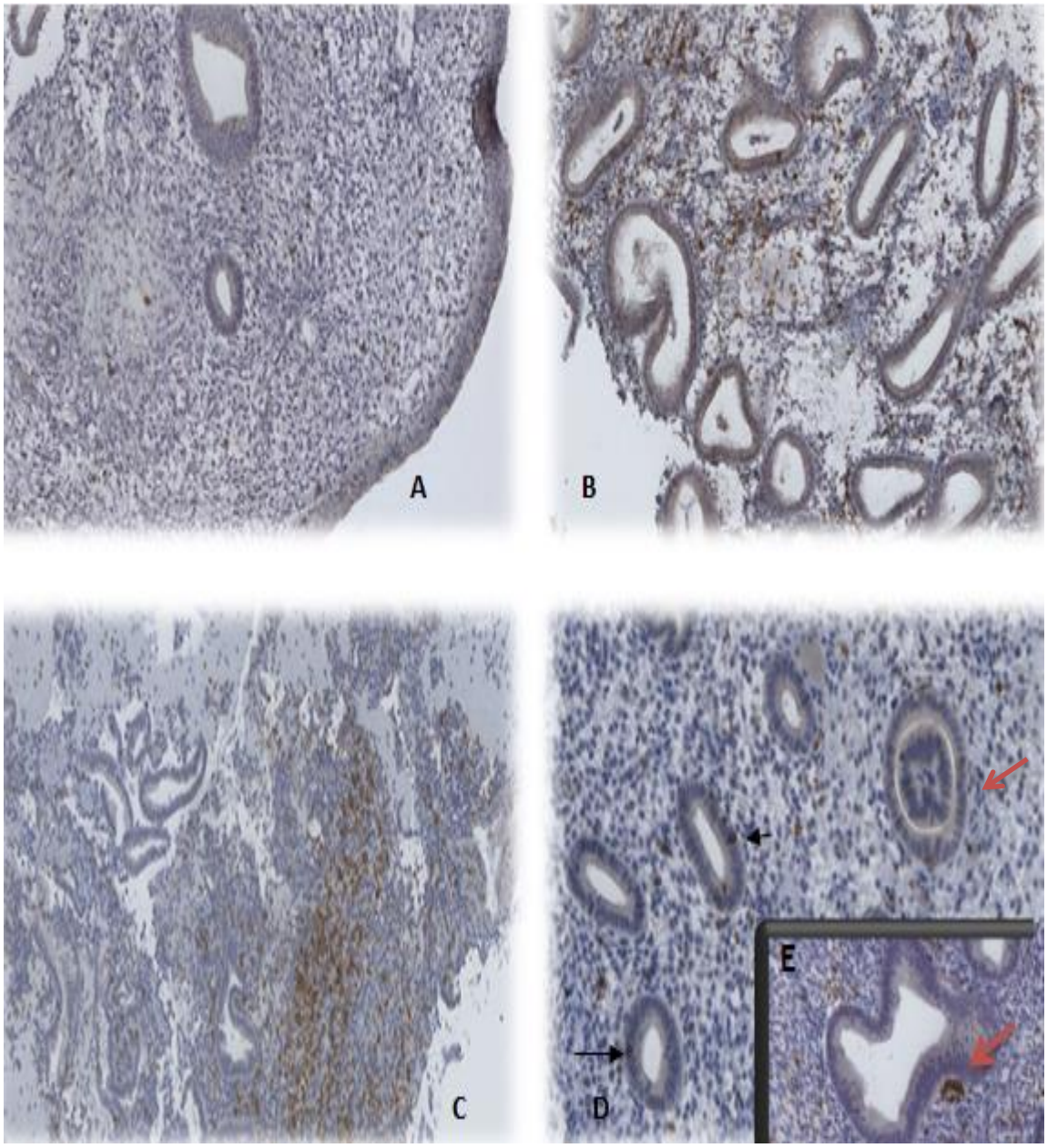


Figure 4.4: Immunolocalization of CD56 (uterine natural killer) cells in endometrium of women with heavy menstrual bleeding. The expression of CD56+ cells is in stromal cells across the menstrual cycle; proliferative (A), secretory (B) and menstrual (C). The infiltration of CD56+ cells (cells in brown colour) in stromal cells, are singular or in aggregation (E), and sometimes immune-positive CD56 cells (brown colour) were found interepithelial cells (D). Immune-negative CD56 cells in stromal and glandular epithelial endometrium tissue presented in blue colour.

4.3.3 **CD68 +cells (macrophages)**

The infiltration of CD68+ cells was increased across the menstrual cycle, and it was higher in secretory phase than the proliferative phase. However, there was no difference between mid-secretory, late-secretory and menstrual phases (Figure 4.5). It was noticed that stromal infiltration of CD68 was concentrated mainly in area around the endometrial glands (Figure 4.6).

Our immunostaining data found no difference in the endometrial expression of CD68 between women with uterine fibroids and women with normal uteri.

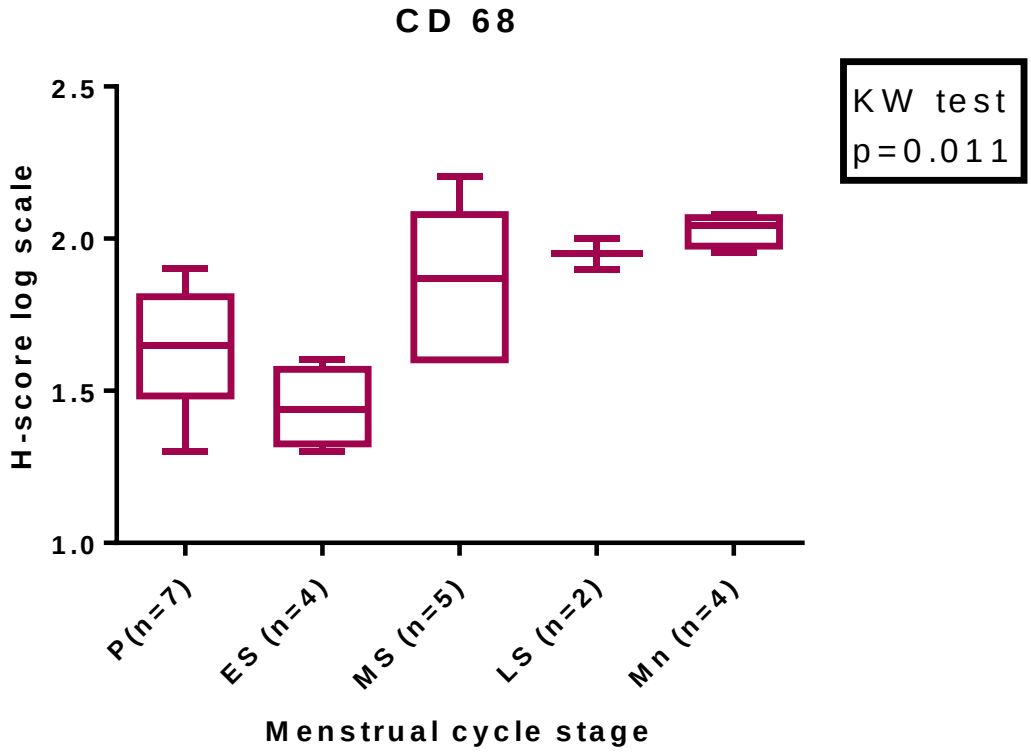


Figure 4.5: Immunostaining score of CD56 in endometrial tissue across the menstrual cycle,P (proliferative), ES (early secretory), MS (midsecretory), LS (late secretory), and Mn (menstrual) phases. (Log scale is depicted).

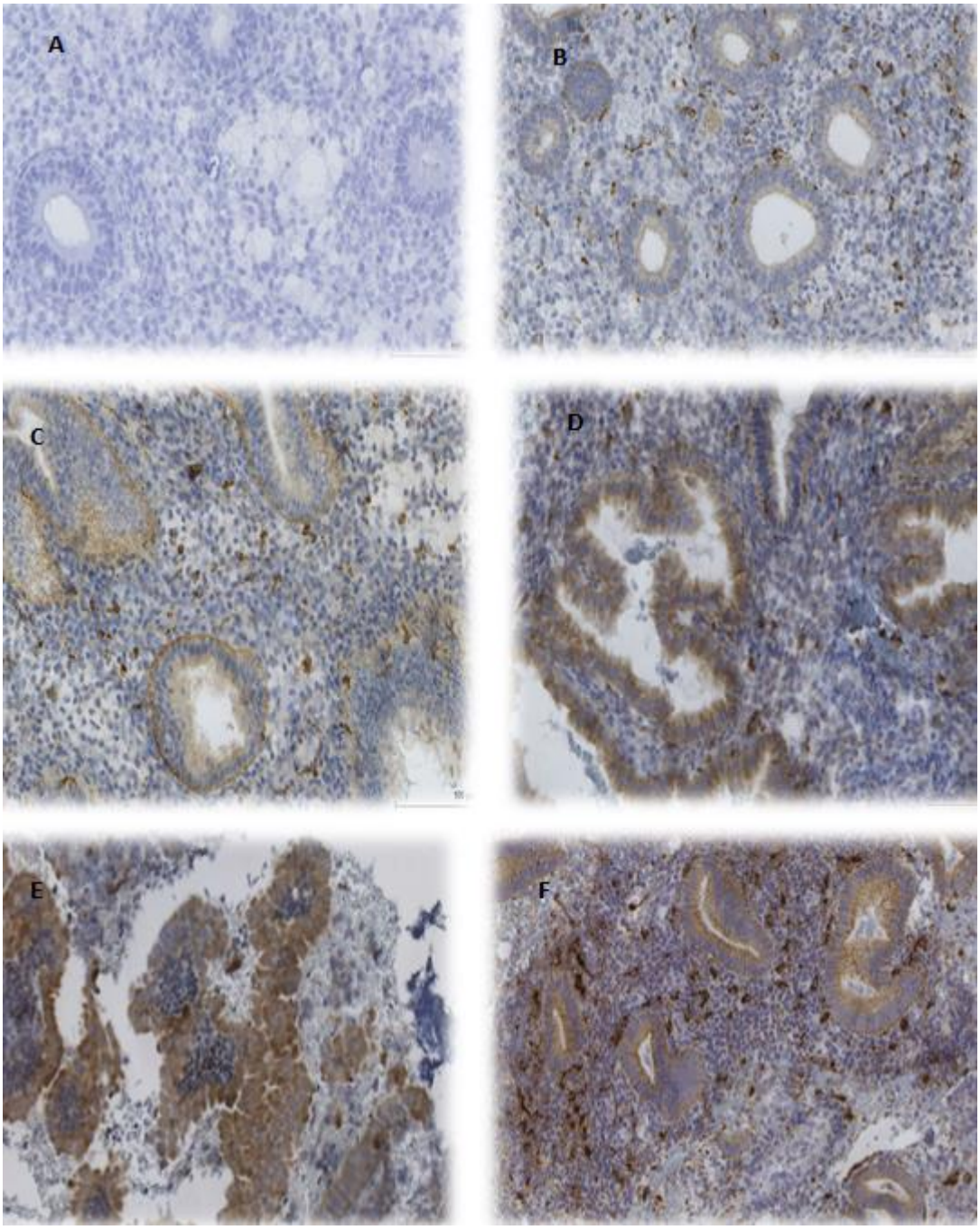


Figure 4.6: Immunolocalisation of CD68 (a specific marker for macrophages) in endometrial biopsies obtained from women with heavy menstrual bleeding. immnuopositive stromal cells presented in brown colour and they infiltrated in the endometrium across the menstrual cycle; proliferative (B), early secretory (C) and mid-secretory phases (D),late secretory (E) and menstrual (F). (A) represents a negative control staining(only blue colour).

4.4 Discussion

Menstruation is one of the endometrial functions which are under the control of a complex interaction between the endocrine and immune systems. Ovarian hormones influence a variety of immune factors within the endometrium to prepare it for implantation or menstruation. Endometrial leukocytes play a crucial role with cytokines and growth factors in tissue breakdown and repair. In other words, the leukocyte may not only influx into endometrium with assistance of many cytokines; they may also have the ability to release many cytokines themselves. In addition the coordinated sequence of inflammation and resolution is important in the regulation of menstruation. Therefore, abnormal inflammatory pathways may impact on the amount of menstrual bleeding. This abnormal inflammatory pathway is also present in endometrium of women with normal uteri and in endometrium of women with uterine fibroids as well as.

4.4.1 **Neutrophils**

Our finding indicated that neutrophil elastase, a key marker for neutrophils, is localised in the stromal tissue. In addition, although it was expressed to an extent throughout the menstrual cycle, it markedly increased during the late secretory and menstrual phase. This result concurred with previous reports[25, 278]. In addition, it may indicate that neutrophil immigration into the endometrium is mainly in response to progesterone withdrawal in the late secretory phase. Decidualization is accompanied by an increased number of leukocytes[279]. If no pregnancy occurs, the ovarian corpus luteum regresses and secretion of both steroids declines at the late secretory phase, triggering extensive infiltration by neutrophils, proteolytic breakdown and shedding of the endometrium with associated bleeding at menstruation[33, 280, 281].Thus the increase in neutrophils within the endometrium coincides with the upregulation of IL8 also in the late secretory phase, where the latter is associated with neutrophils chemotactic function[68]. Stretching of the myometrium in presence of uterine fibroids is a mechanical factor that may stimulate neutrophil activation and adhesion. In addition, it may promote transendometrial migration of neutrophils; this mechanical mechanism can stimulate cytokines production as IL8.

4.4.2 **Uterine killer cells**

The relative number of CD56+ cells (uNK) was increased across the menstrual cycle with higher infiltration during the mid and late secretory phase. This is in agreement with Bulmer et al [254, 279]and Kitaya et al [282]. This post-ovulatory increase in the relative number of endometrial uNK cells suggests that these cells play a role in uterine events such as pregnancy and menstruation. In addition, it may indicate involvement of steroids hormones, particularly progesterone in the post-ovulatory rise of uNK cells. However, uNK cells do not express progesterone receptors but do express the prolactin receptor, oesterogen receptor ß and glucocorticoid receptor [18, 24]. In a previous report, where an increase in the uNK cells was noted in endometrium of postmenopausal women having hormonal replacement therapy[283], which supports this concept. Nevertheless, aberrant numbers of uNK cells in the mid-secretory phase was associated with implantation failure and recurrent miscarriages [284, 285]. In parallel with other organs, the increase of uNK cells in the postovulatory period seems to result from the expansion of two NK cell populations with different origins; one is in situ proliferation of the resident uNK population, and the other is the selective influx of a population from the endometrial microcirculation[286]. The uNK cells may be able to promote angiogenesis as they express some growth factors that are essential for angiogenesis such as vascular endothelial growth factor[287], placental growth factor, angiopoeitin 2, and NKT5[288]. A large number of uNK cells has been found in decidua in early pregnancy, and it has been proposed that uNk cells have a role in regulating extravillous trophoblast invasion and spiral artery remodelling[289]. In non-conceptual menstrual cycle the number of uNK has been reported as either- small or absent in the proliferative and early secretory phase[290] and high in mid-late secretory phase. Therefore, as discussed above, increases in the number of uNK in mid-late secretory phase endometrium can cause dysregulation of angiogenesis which may attribute and associated with heavy menstrual blood loss. In this study it was noticed that the relative number of uNK cells was higher during mid-late secretory phases than the menstrual phase, however, the difference was not significant. This may suggest a decrease in the apoptosis rate of the uNK cells which habitually show a high rate of

apoptosis two to three days before menstruation [291, 292]. If pregnancy occurs, uNK cells are upregulated and have the highest infiltration into the decidua of early pregnancy. Our results suggest upregulation of uNk in heavy menstrual bleeding endometrial stroma during secretory and menstrual phases, potentially indicating a role of uNK in this condition.

4.4.3 **Macrophages**

The immunohistochemistry data showed a high number of CD68 +cells (key marker of macrophages) in proliferative phase and reach the peak in mid-late secretory and menstrual cycle phases. This is the same pattern of infiltration of CD68 +cells in nomal endometrium during the menstrual cycle [220, 226, 227]. However, the increase in the relative number of macrophages in endometrial stroma has been found to be associated with menstrual abnormality [229]. The relative number of macrophages in endometrium is well regulated, temporally and spatially during the menstrual cycle. Increased macrophage number across the menstrual cycle in women with heavy menstrual bleeding is considered to occur by recruiting monocytes from the peripheral circulation and/ or in situ proliferation within endometrium [20, 293]. Presence of macrophages in the proliferative phase suggests a potential role in regeneration and proliferation of endometrium. This cell function may undergo oestrogen-dependent regulation. On the other hand, the high number of macrophages during the mid-late secretory phase of the menstrual cycle is assumed to suggest specific roles in regulation of fertility. Macrophages have been identified as important course for pro-inflammatory and anti-inflammatory chemokines. Therefore, upregulation of macrophages in endometrium across the menstrual cycle may enhance the proinflammatory environment in endometrium and as a consequence , dysregulation of local endometrial hemostatic mechanisms takes place [294]. Unscheduled bleeding is thought to be the most common reason for discontinuation of contraceptives [295, 296]. A previous report showed an increase in macrophages and their cytokines in the endometrium of women using a LNG contraceptive who complained of unscheduled bleeding[229]. Further studies using progestin-only contraceptives have also shown an increase in endometrial MMPs [297, 298]. These observations highlight the importance of dysregulation of macrophage numbers in the context of endometrial bleeding

patterns. However, as a role for macrophages in regulation of menstrual function is yet to be defined, the actual impact of dysregulation of macrophage function and number on endometrial bleeding remains to be determined.

4.4.4 Leukocytes in women with uterine fibroids

In the present study, there was no difference in the infiltration of the leukocytes studied into endometrial stromal tissue in the presence of uterine fibroids. This may be because most of the endometrial biopsies were from women with intramural uterine fibroids where the uterine cavity was not distorted. In addition, there is a concept that variation in the endometrial size due to the use of clinical biopsies in the research may obscure the differences between the two groups, since the total number of stromal cells was very low in some samples. It is important to determine the minimum cell count required to be examined to produce a precise result, which is broadly equivalent to counting stromal cells in ten microscopic non overlapping fields at a magnification of 400×. However, to our knowledge, in the studies carried out to date, leukocytes in tissue section were identified by immunohistochemistry and the number of cells expressed as a relative number of the total stromal cells. On the other hand, some of these findings are conflicting. For instance, in one report the reference range of uNk cells in normal endometrial varied from 0.2 to 9.5% of stromal cells [299]and from 2.2 to 13.9 in another study[300].

4.5 Conclusion

It seems that the pattern of leukocyte infiltration in endometrium of women with regular heavy menstrual bleeding parallels with that in endometrium of normal subjects. However, there is upregulation and increase in the number of leukocytes across the menstrual cycle which may contribute to heavy blood loss at menstruation. In presence of uterine fibroids, the recruitment of leukocytes is not different especially when there is uterine cavity distortion.

Chapter 5:

5. Theexpression of VEGF-A in uterine tissue in women with HMB in presence of uterine fibroids

5.1 Introduction

The human endometrium undergoes remarkable cyclic growth and regeneration in response to various environmental changes, such as hypoxia or complex interactions of the female sex steroids during the menstrual cycle. A new vascular system develops via angiogenesis and vascular remodelling in the endometrium during each menstrual cycle to support cellular growth and differentiation[33, 301]. Angiogenesis and vascular remodelling are believed to be orchestrated by coordinated interactions of several angiogenic factors in the human endometrium [302].Vascular endothelial growth factor (VEGF), stimulates endothelial cell proliferation, permeability, migration, and assembly into capillary tubes [303, 304]. Most work regarding the regulation of endometrial angiogenesis has focused on VEGF [305, 306]. Recent studies have reported that VEGF is essential for the rapid burst of angiogenesis that occurs during postmenstrual repair and early proliferative phases in the primate endometrium and furthermore plays a role in re-epithelialisation of the endometrium[303].

5.1.1 Vascular endothelial growth factor isoforms and receptors

Vascular endothelial growth factor is a mitogenic signal protein which works mainly on the endothelial cells [307, 308]. Through alternative mRNA splicing, a single gene gives rise to several distinct isoforms of VEGF, these different isoforms of VEGF have been identified according to their amino acid residue length (VEGF121-VEGF165,VEGF189,and VEGF206) [309-311]. These isoforms differ in their expression patterns as well as their biochemical and biological properties [307]. VEGF165 is the predominant isoform[304], and it is produced by normal and transformed cells. Less frequent splice variants have been also reported, such as VEGF145 and VEGF183[312]. In the past the term VEGF was used to indicate to VEGF-A which is one of the vascular endothelial family. In present this family includes VEGFA, B,C,D,E and placenta growth factor (PlGF)[313]. Two VEGF receptor tyrosine kinases (VEGFRs) have been identified, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). VEGFR-2 seems to mediate almost all observed endothelial cell responses to VEGF, whereas roles for VEGFR-1 are more elusive. VEGFR-1 might act predominantly as a ligand-binding molecule, sequestering VEGF from VEGFR-2 signalling. In addition, there is a vascular

endothelial growth factor receptor-3 (VEGFR-3, Flt-4), a receptor for vascular endothelial growth factors (VEGFs) C and D, is specifically expressed on lymphatic endothelium and suggested to play a role in the maintenance of lymphatic endothelium and/or in lymphangiogenesis [314] (Figure 5.1).

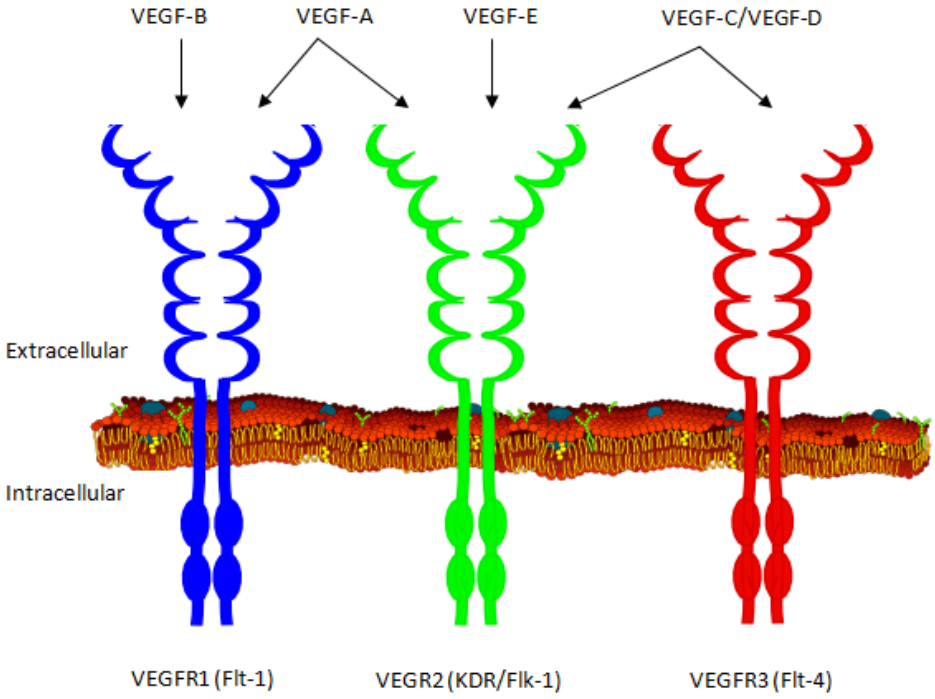


Figure 5.1: Vascular endothelial growth factor isoforms and receptors are depicted above, with different colours referencing different ligands of VEGF have different mediate function. Both VEGFR1 and VEGFR2 bind to VEGF-A.

5.1.2 Expression of vascular endothelial growth factor in human endometrium

The expression of VEGF-A was determined in all cell types in the human endometrium and in all phases of the menstrual cycle [315, 316]. There is a strong belief that VEGF production is induced mainly by hypoxia and as a consequence, the expression of VEGF is elevated during menstruation [315,317, 318]. VEGF-A is also increased in the mid-secretary phase [306, 317], which is

most likely because of concerted actions of estradiol (E2) and progesterone [319-324]. These studies clearly indicate that VEGF-A production in human endometrium is stimulated by oestrogen. On the other hand, some other studies found elevated expression of VEGF-A in the proliferative phase [94,325,326]. It has also been suggested that VEGF-A production by cultured endometrial tissue is high in peri and post menstrual human endometrium [313, 327], indicating to high level of VEGF-A during menstruation and the early proliferative phase.

5.1.3 **Vascular endothelial growth factor role in angiogenesis**

Angiogensis is an essential mechanism to support endometrial growth after menstruation and to provide a vascularised, receptive endometrium for implantation and placenta formation.The vascular growth mechanism in the human endometrium and the time frame of various processes included in this mechanism are still unclear[328]. Nevertheless, it thought that this mechanism is regulated by a combination of systemic and local factors, where their actions are coordinated by 17B-estradiol and progesterone and most likely mediated by locally acting factors[10]. The vascular endothelia growth factor family is a major mediator of angiogenesis [326] and VEGF-A plays the main role in angiogenesis induction and vascular remodelling [10, 329-331]. In addition, it plays a crucial role in endothelial cells survival by providing defence action againstapoptosis induced by various pro-apoptotic agents [332, 333]. It was found that VEGF-A can stimulate the migration and the proliferation of endothelial cells of blood vessel origin and promotes blood vessel angiogenesis and increases vascular permeability in vitro [303, 304, 334, 335]. However, VEGF-A may induce proliferation and increase the survival of epithelial cells in an autocrine manner [336], and there are also indications that VEGF-A is involved in epithelial cell function[313].

5.1.4 **Vascular endothelial growth factor and menstrual cycle**

The endometrium undergoes regular cycles of growth and breakdown and is one of the few adult tissues in which significant angiogenesis occurs on an ongoing, physiological basis progressing from vessel stumps in the basal layer of endometrium that remain after menstruation[78]. After menstruation , hypoxia

enhances angiogenesis by inducing human endometrial stromal cells to express VEGF [337], thus, VEGF regulates epithelial and stromal development in endometrium under the influence of oestrogen and progesterone, thereby it regulates the growth and differentiation of endometrium during the menstrual cycle[303]. Abnormal angiogenic process may lead to menstrual abnormality as well. This abnormal angiogenesis reflects the progestational inhibition of endometrial blood flow promoting local hypoxia and the generation of reactive oxygen species that increase the production of angiogenic factors such as vascular endothelial growth factor in human endothelial stromal cells[338].This was supported by some data that showed that expression of vascular endothelial growth factor (VEGF)-A and its two main receptors, VEGFR-1 and -2, is increased in idiopathic heavy menstrual bleeding. Nevertheless, another study found that VEGF-A level was higher in women with normal menstrual cycles than that for women with heavy menstrual bleeding [94].

5.1.5 **Vascular endothelial growth factor in myometrium and uterine fibroids**

The myometrium is the muscular wall of the uterus that undergoes important changes in size and cellular properties in many physiological and pathological conditions. The myometrium undergoes some changes during each reproductive cycle [339] and remarkable modifications throughout pregnancy [340] as well as menopause [341]. The uterine growth during pregnancy represents one of the most remarkable events in reproduction, with a massive increase in both size and number of myometrial smooth muscle cells, to allow the growing fetus to have the necessary support. Myometrial mass and cellular morphology are also modified in tumour conditions such as leiomyosarcoma and uterine fibroids [342, 343].Uterine fibroids are the most common gynaecologic tumours that occur in women of reproductive age, but their molecular pathogenesis is still unknown. Since the growth of uterine fibroids involves several vascular factors, an association between uterine fibroids and growth factors is suspected [344, 345]. A number of growth factors, including transforming growth factor-ß (TGF-ß), epidermal growth factor (EGF), insulin-like growth factors 1 (IGF-1)and 2 (IGF-2), basic fibroblast growth factor(bFGF), platelet-derived growth factor(PDGF), have been detected in myometrium and uterine fibroids[346, 347]. These growth

factors have a main role in the process of growth and differentiation of uterine fibroids [348]. It has been found by several studies that IGF-1 is the most infiltrated growth factor in uterine fibroids [349-351].

The role of vascular endothelial growth factor (VEGF) in human endometrium has been studied by many authors. It is known, as described above, that VEGF regulates a number of endometrial functions. However, the role of VEGF in myometrium is not adequately identified. The angiogenic role of VEGF in malignant tumour growth stimulation was well documented in several studies [352-354]. This stimulating action includes enhancing both new angiogenesis and branching of already existing arterial and capillary vessels[331], and promoting the proliferation of tumour cells and facilitate the metastasis process [354, 355]. Many authors reported the expression of VEGF in uterine fibroids [354, 356]. However, the function of VEGF in uterine fibroids is not clear since it is rare for uterine fibroids to transfer to malignancy, and since oestrogens stimulate VEGF production in endometrium [324]. Thus, these hormones may promote fibroid growth by the stimulation of angiogenesis.

Because of the growth of uterine fibroids is regulated by ovarian steroids, therefore, low estrogenic state, similar to menopause, is associated with the decrease of uterine fibroid volume. The same effect is obtained with the administration of GnRH-agonist (GnRH-a). where it reversibly suppresses the ovarian function, lowering estradiol levels to menopausal values and causing a decrease in uterine volume and fibroids[357]. GnRH-a also help to control uterine bleeding that are the most common symptoms associated with uterine fibroids. After the cessation of GnRH-aadministration, uterine fibroids regain their original size, so that in premenopausal women therapy is considered as a preoperative treatment.

5.1.6 **Aims**

Many studies have focused on the patterns of VEGF-A gene expression in human endometrium in normal and heavy menstrual bleeding. This study focuses on the transcriptional expression patterns of VEGF-A and its main receptors in endometrium from women with heavy menstrual bleeding and uterine fibroids and with normal uteri. It aims to determine if there are any differences in VEGF-

A expression between the two groups. In addition, the study focuses on the expression of the same markers in myometrium and uterine fibroids.

5.2 Methods

5.2.1 Subjects

The studied subjects were women aged from 39-51 years (premenopausal age) with regular menstrual cycles (from 21 to 35 days) who presented to the outpatients clinic complaining of heavy menstrual bleeding (HMB) in presence of uterine fibroids or no uterine pathology. Subjects had not received hormonal preparation in the 3 months preceding biopsy collection (group 1&2).Women receiving hormonal therapy comprise a separate group (group 3). In this chapter three subject groups were studied. Ethical approval was obtained from West of Scotland REC 5 research ethics committee and written informed patient consent obtained before tissue collection. Menstrual blood loss was assessed using the Pictorial Blood Loss Assessment Chart (PBAC) as in chapter 3.

5.2.1.1 Group 1

This group comprised women who attended a hysteroscopy clinic at Stobhill Hospital, Glasgow and had hysteroscopy as a part of their heavy menstrual bleeding investigation. Some had no uterine pathology and others had uterine fibroids. Uterine fibroids were diagnosed by either transvaginal ultrasound or hysteroscopy.

5.2.1.2 Group 2

Women had hysterectomy at the gynaecology department, Glasgow Royal Infirmary (GRI) as treatment for their uterine fibroids. Uterine fibroids were diagnosed by either transvaginal ultrasound or magnetic resonance image (IMR).

5.2.1.3 Group 3

Women had gonadotrophin releasing hormone injection within 2 to 3 months before hysterectomy for uterine fibroids (n=3).

5.2.2 Tissue collection

Endometrial samples were obtained from group 1 by means of Pipelle sampler, while in group 2 &3, endometrial, myometrial and fibroid tissues were collected by pathologists. See section 2.2.2 for biopsies handling and storage).

Endometrial biopsies were dated according to Noyes criteria (Noyes et al., 1975) by a pathologist (Prof. Alistair Williams, University of Edinburgh) and the reported last menstrual periods (LMP) (Table 5.1). Circulating estradiol and progesterone concentrations were measured at the time of endometrial and uterine biopsies and were consistent with the histological assessment (Table 5.2).

Table 5.1: Summary of endometrial biopsies collected from women with uterine fibroids and women with no uterine pathology that had provided the Pictorial Blood Loss Assessment chart back.

Pathology	P	ES	MS	LS	Mn	Inactive /excluded	Inadequate for staging
Uterine fibroids	10	8	4	1	5	4	3
normal uteri	8	4	5	2	6	5	1

Table 5.2: Serum estradiol (E2) and progesterone (prog.) at the time of endometrial biopsy

	Uterine fibroid cases		Normal uteri case	
	Mean E2 (range) (pmol/l)	Mean prog.(range) (nmol/l)	Mean E2 ±SE (pmol/l)	Mean prog.± SE (nmol/l)
Menstrual	49.40(37-72)	4.84(0.4-21)	106.7(65-191)	1.22(0.6-1.8)
proliferative	329(73-908)	1.54(0.3-3.9)	575(105-3180)	0.55(0.3-1.0)
Early secretory	399.4(211-830)	18.81(0.3-36)	702(192-1343)	15.58(0.4-32.6)
Mid secretory	279(166-389)	9.78(0.3-26)	394.8(141-575)	28.40(9.0-52)
With GnRH agonists	38.3(37-176)	0.43(0.3-0.5)		

5.2.3 **Immunohistochemistry**

To investigate the expression of the VEGFA in human endometrium and uterine tissue, 3 µm paraffin-embedded sections were de-waxed and rehydrated through graded alcohols before endogenous peroxidase activity was blocked by 30 minute incubation in 0.5%solution of hydrogen peroxide and methanol. The sections were incubated for 30 minutes with normal blocking serum (20% horse serum and 20% human serum) before being incubated over night at 4 C° with the primary antibody polyclonal goat anti- human VEGF-A (Santa Cruz Biotechnology, SC-152-G) at 1:200 dilution. Sections were then incubated with the secondary antibody (biotinylated anti-goat IgG (H+ L) AB- 9500 made in horse) after washing in phosphate buffer solution (2x 5minutes), and a corresponding post-primary protocol (section 2.6.5). Sections were counterstained in haematoxylin

and mounted in pertex. Human kidney tissue was used as positive control for optimising immunohistochemistry protocol (Figure 5.2).Sections were scanned by Claire Orange using slide path technique for scoring method.

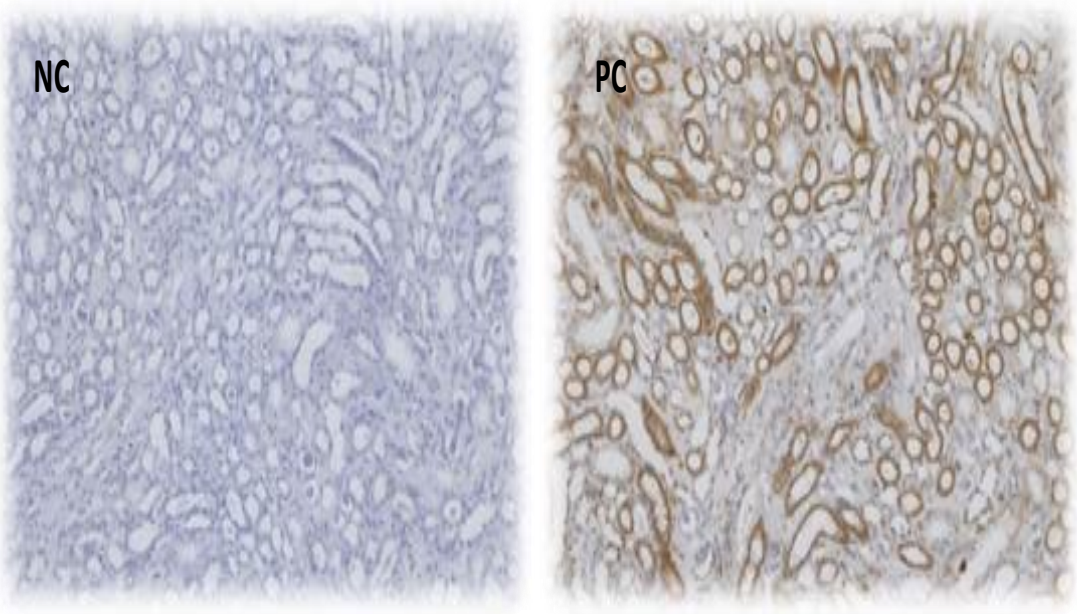


Figure 5.2: Human kidney tissue used as a positive control for VEGF-A immunohistochemistry, the brown stained tissue.

5.2.4 **Quantitative RT-PCR**

RNA was extracted with TRI-reagent (Ambion, life technologies, Paisley, Uk) following manufacturers guidelines using phase lock tubes (Microcentrifuge, greiner bio-one GmbH, Stone House- England, UK). RNA samples were reverse transcribed using MultiScribe (Invetrogen, Life technologies, Paisley, UK) primed with random hexamers according to the manufacturer's instructions (see section 2.5.4). where there was no enough fresh endometrial samples , RNA was extracted from formalin –fixed paraffin- embedded samples using Ambien kit 1975 (Recover All total nucleic acid isolation kit-Life technology, Paisley, Uk) according to the manufacturer's instructions (see section 2.5.1.2). Quality of RNA

was assessed using an automated spectrophotometer RNA 6000 Nanodrop (Lab tech.com, Ringmer, East Sussex,UK), only half of the RNA samples were assisted by the Agilent 2100 Bioanalyser system in combination with RNA 6000nano chips (see details in section2.5.2).Once the RNA was extracted and quantified, the PCR reactions were carried out using an ABI Prism 7900 (Applied Biosystems) as previously described in section 2.5.6 using duplicate samples. A no template control (containing water) was included. Primer and FAM (6-carboxyfluorescein)-labelled probe are shown in Table 2 (section 2.5.4). Multiple transcript variants have been reported for human VEGF and alternative mRNA splicing. a single gene gives rise to several distinct isoforms of VEGF, which differ in their expression patterns as well as their biochemical and biological properties[307]. Three VEGF receptor tyrosine kinases (VEGFRs) have been identified, VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) [307, 358]and VEGF-R3 (Flt-4)[359].The primer utilised in this study did not distinguish between these isoforms. Pre-validated primers and probes were purchased for VEGF-A, VEGF-R1, and VEGF-R2 (Life technology, Paisley,Uk). Gene expression was normalised to B-actin ribosomal RNA (Applied Biosystems) as an internal standard. Data were analysed and processed using Sequence Detector System (SDS version 2.3) according to the manufacturer's instructions. Results are expressed as relative to a standard cDNA obtained from a single sample of endometrial tissue and included in all reactions.

5.2.5 **Enzyme-linked immunosorbent assay (ELISA)**

The concentration of vascular endothelial growth factor (VEGF) in serum was measured by ELISA. The component required for this procedure was supplied in the quantikine kit (R&D Systems Europe, Ltd, Abingdon, UK).the quantitative immunoenzymometric sandwich technique was followed according to the manufacturer's instructions. A monoclonal antibody specific for VEGF was coated onto the microtiter plate provided in the kit. Standards with known amount of the cytokine and samples were pipetted into the wells and any VEGF was bound by immobilised antibody. Any unbound sample protein were washed away and an enzyme linked polyclonal antibody specific for VEGF was added to the well and allowed to bind to the VEGF which was bound during the first incubation. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution was

added to the wells and a colour developed in proportion to the amount of VEGF which was bound in the initial step. The colour development was stopped and the colour intensity was measured using spectrophotometer. A curve was prepared, plotting the optical density (OD) versus the concentration of VEGF in the standard wells. By comparing the OD of the samples to the standard curve, the concentration of VEGF in unkwon samples was determined.

5.2.6 Immunohistoscoring and analysis

Scoring of immunostaining intensity of VEGF-A in stromal endometrial tissue was subjected to histoscoring method, while the immunostaining intensity of the VEGF-A in glandual endothelial tissue was assessed in a semi-quantitative manner on a 4-point scale: 0=no immunostaining, 1= mild immunostaining, 2= moderate immunostaining, and 3=intense immunostaining. 90% of tissue sections were measured with a computerised image analysis system (Version 4.0, Digital Image Hub, Leica biosystems), and 10% of them was scored by the research fellow, a strong correlation between scores derived from the image analysis and subjective scores by the observer was obtained.

5.2.7 Statistics

Data were subjected to statistical analysis using Mann-Whitney test or Kruskall-Wallis for comparison between groups, and Spearman correlation test. Minitab statistical 16 software was used for statistical analysis and graph pad prism 6 software was used for creating Figures.

5.3 Results

5.3.1 VEGF-A protein expression within the human endometrium across the menstrual cycle

Standard immunohistochemical technique successfully demonstrated the expression of VEGF-A protein in both stromal and glandular epithelial compartments of endometrium (Figure 5.3&5.4). In addition, VEFG-A protein expression was demonstrated around blood vessels (Figure5.5).

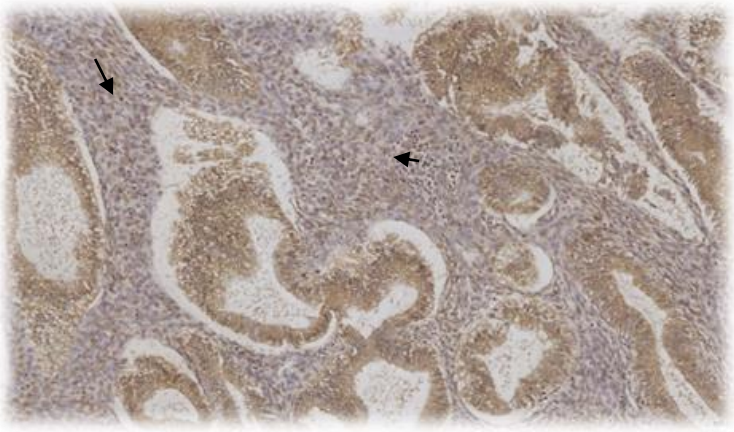


Figure 5.3: The expression of VEGF-A protein in endometrial stromal cells (arrow). The brown stained cells are positive for VEGF-A and blue stained cells are negative for VEGF-A.

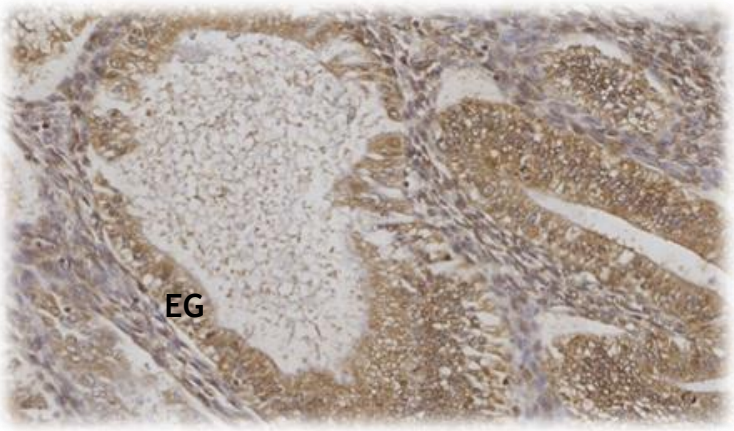


Figure 5.4: The expression of VEGF-A protein in endometrial epithelial glandular tissue (EG). The brown stained cells are positive for VEGF-A and blue stained cells are negative for VEGF-A.

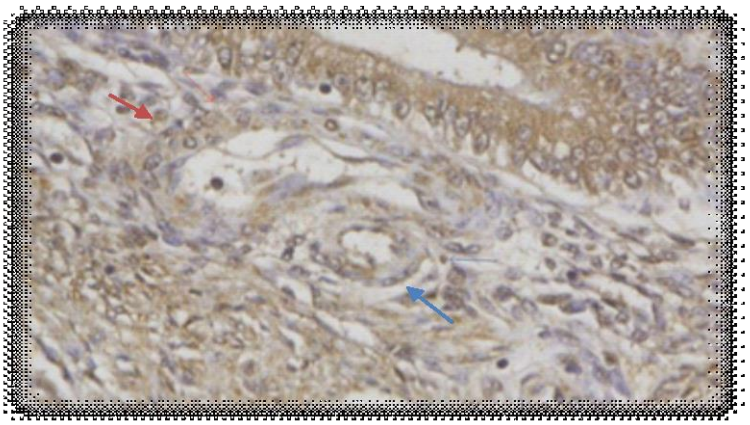


Figure 5.5: VEGF-A around arteries (red arrow) and veins (blue arrow) in human endometrium. The brown stained cells are positive for VEGF-A and blue stained cells are negative for VEGF-A.

5.3.1.1 In women with normal uteri

In stromal endometrial tissue, cellular expression of VEGF-A protein appeared higher in the nuclear compartment than the cytoplasmic compartment. However, the immunostaining analysis found no statistic significant variation in VEGF-A protein expression between cellular compartments. The pattern of VGEF-A protein expression in stromal endometrium displayed a highest infiltration level in the menstrual phase and the lowest level during the early secretory phase, with significant differences between the two levels (95% Cl (129.99, 30.00) P= 0.019). The expression of VEFG-A protein was higher in the proliferative phase than the early secretory phase as well (95% Cl (99.97, 0.02) p=0.04).In glandular endometrial tissue, the VEGF-A protein infiltration was higher in the menstrual phase than the secretory phase (95% Cl (0.199, 1.686) p= 0.019) (Figure5.5).

5.3.1.2 In women with uterine fibroids

The pattern of VEGF-A protein expression in stromal endometrial increased across the menstrual cycle with no significant difference between phases.It was noticed that VEGF-A protein staining intensity in glandular endometrial tissue significantly increased throughout the menstrual cycle from the proliferative phase toward menstrual phase for women with uterine fibroids (P= 0.001)(Figure 5.6).

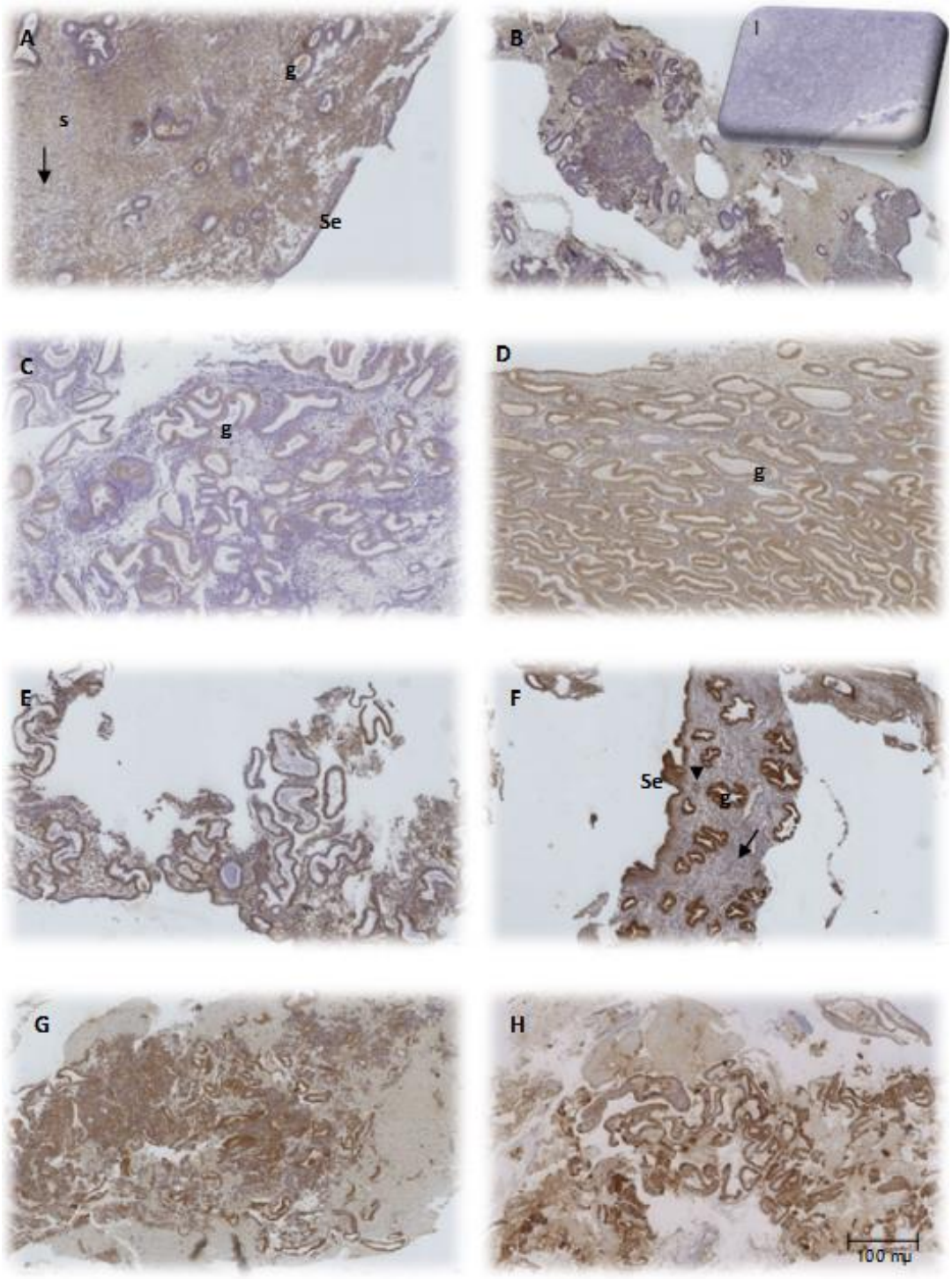


Figure 5.6: : Representative sections of immunostaining of VEGF-A proteins a cross the menstrual cycle in women with normal uteri, proliferative(A), early secretory (C), mid-secretory(E), and menstrual phase(G), in women withuterine fibroids, proliferative (B), early secretory (D), mid-secretory (F) and menstrual phase (H). In each section VEGF-A found in epithelial glands (g), and stroma (s) surface epithelial (Se) and in endometrial capillaries (arrows). Negative control tissue (I). The immunostaining positive cells for VEGF-A presented in brown colour and the immunostaining blue cells are negative for VEGF-A.

5.3.1.3 Differences in endometrial VEGF-A protein expression between women with uterine fibroids and women with normal uteri

There was no difference in the VEGF-A protein expression throughout the menstrual cycle in the stromal endometrium between women with normal uteri and women with uterine fibroids except in the early secretory phase (97% Cl (-130.03, 0.00), p=0.043), where it was higher in endometrial samples for women with uterine fibroids(Figure 5.7). The difference in VEGF-A protein in glandular tissue between the two groups was only not significant P=0.587.

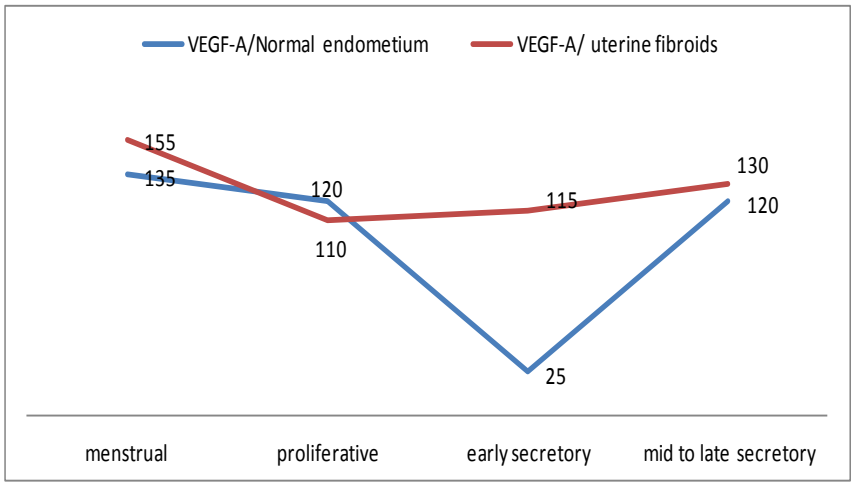


Figure5.7: the expression pattern of VEGF-A protein in the stromal endometrial throughout the menstrual cycle for both women with uterine fibroids and women with normal uteri (medians value was depicted). Proliferative phase n=9&7, early secretory phase n=8&4, mid-late secretory phase n=5&7, and menstrual phase n=4&5 for uterine fibroids group and normal uteri group respectively.

5.3.2 Expression of VEGF-A mRNA across the menstrual cycle

5.3.2.1 In women with normal uteri

Endometrial VEGF-A mRNA expression varied significantly across the menstrual cycle in women had HMB with normal uteri (p=0.006) (Figure 5.8). However,no difference in the expression of VEGFA was found between the proliferative and

mid- late secretory phase. The variation in the VEGF-A mRNA levels was consistent with immunostaining intensity results.

5.3.2.2 In women with uterine fibroids

The RT-PCR results show that the relative VEGF-A mRNA expression was significantly higher in the proliferative phase and mid-late secretory phase than the early secretory phase, with no difference between the proliferative and mid-late secretory phase (Figure 5.8).

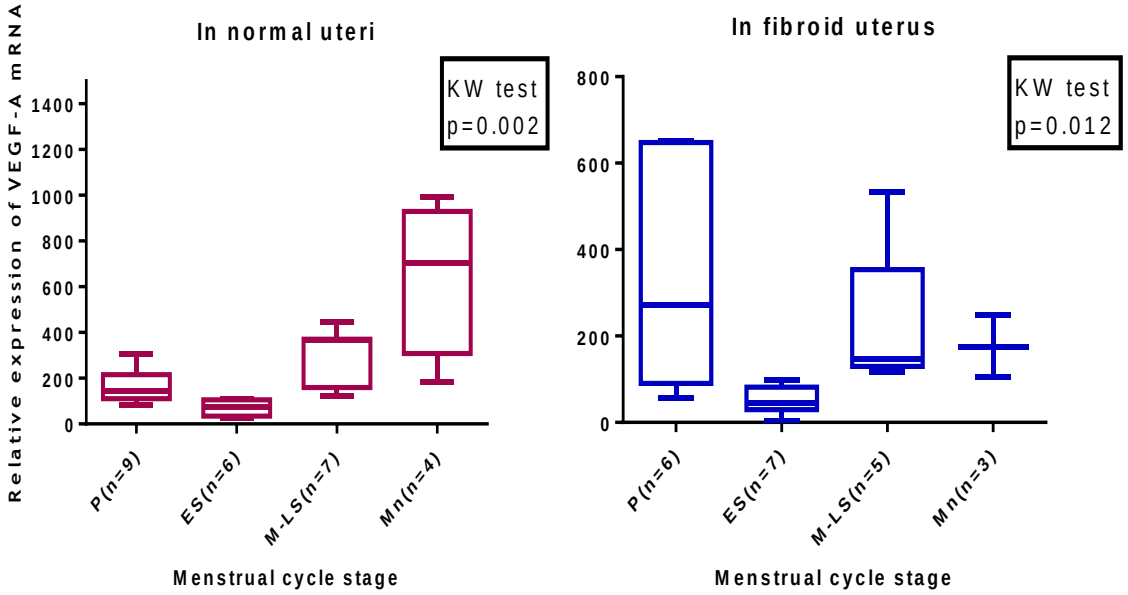


Figure 5.8: VEGF-A mRNA expression across the menstrual cycle in women had heavy menstrual bleeding (HMB) with normal uteri and with uterine fibroids;proliferative (P), early secretory (ES), mid-secretory (MS), late secretory (LS) and menstrual (Mn) phases. The actual values of the relative expression of VEGF-A mRNA were analysed by Kruskal Wallis test.

5.3.2.3 Difference in the VEGF-A mRNA levels between groups

Our RT-PCR data found no difference in the expression of VEGF-A in endometrium for women with HMB between uterine fibroids and normal uteri cases, and this finding was consistent with the immunohistochemistry data (Table 5.3).

Table 5.3: Summary of differences between groups for the relative VEGF-A mRNAexpression, representative in medians (Mann-Whitney test was used).

		Endometrium/uterine fibroids	Endometrium/normal uteri			
Comparison phase	No.	Median	No.	Median	95% Cl	P value
proliferative	6	270.7	9	147.6.6	(499.2.0,108.1)	0.68
Early secretory	7	46.67	6	72.60	(49.71,67.25)	0.77
Mid-late secretory	5	160.5	7	365.0	(327.8,297.8)	0.54
menstrual	3	173.3	4	705.9	(63.4,890.4)	0.11

5.3.3 The expression of VEGF receptors across the menstrual cycle

5.3.3.1 VEGF-R1 (flt-1)

The RT-PCR results for VEGF-receptor 1 (VEGFA-R1) in women had with normal uteri demonstrated that the expression increased from the proliferative across the menstrual cycle and obtained the highest expression in the menstrual phase and the lowest expression in the early secretory phase with no statistical significant difference between the maximum and the minimum expression (Figure 5.9).In women with uterine fibroids, there was no difference in the expression of VEGFR1 across the menstrual cycle (Figure 5.9).

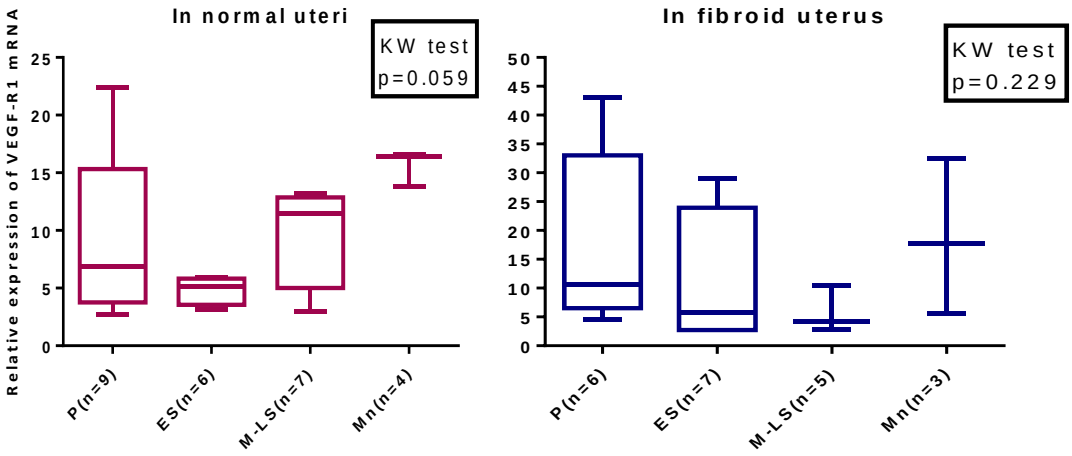


Figure 5.9: Illustration of the differences in the relative expression of mRNA of VEGF-R1 in the endometrium throughout the menstrual phase; in proliferative (P), early secretory (ES), mid to late secretory (M-LS) and menstrual (Mn) for women with normal uteri and women with uterine fibroids. The actual values of the relative expression of VEGF-R1 mRNA were analysed by Kruskal Wallis test

5.3.3.1 VEGF-R2 (KDR)

The analysis of RT-PCR data for VEGF-R2 found that the expression of this receptor in endometrium for women had HMB with no uterine pathology, was significantly increase across the menstrual cycle (Figure 5.10).In women who had HMB with uterine fibroids; there was no any difference in the VEGFR2 expression between phases across the menstrual cycle. Nevertheless, the expression of that receptor increased from the proliferative to the secretory phase until obtained the highest expression in the menstrual phase (Figure 5.10).

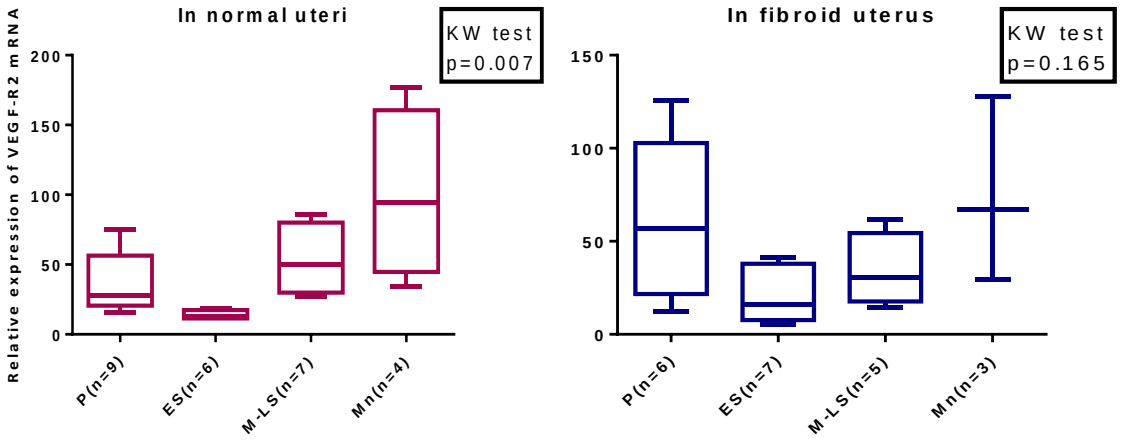


Figure 5.10: differences in the relative expression of mRNA of VEGF-R2 in the endometrium throughout the menstrual phase; in proliferative (P), early (ES), mid to late secretory (M-LS) and menstrual (Mn) for women with normal uteri and women with uterine fibroids. The actual values of the relative expression of VEGF-R2 mRNA were analysed by Kruskal Wallis test

5.3.3.2 Relation between VEGF-A and its receptors (ftl-1&KDR)

The correlation between the VEGF-A and its two receptors was tested and our results found no relation between the mRNA levels of VEGF-A and the receptor VEGF-R1 across the menstrual cycle for both groups, moreover, The mRNA level of VEGF-R2 did not correspond to the level of VEGF-A across the menstrual cycle.

The findings indicated to a higher expression of VEGF-R2 than VEGF-R1 in the proliferative phase ($P<0.0001$), and early secretory ($p<0.05$) for women with HMB (Figure 5.11).

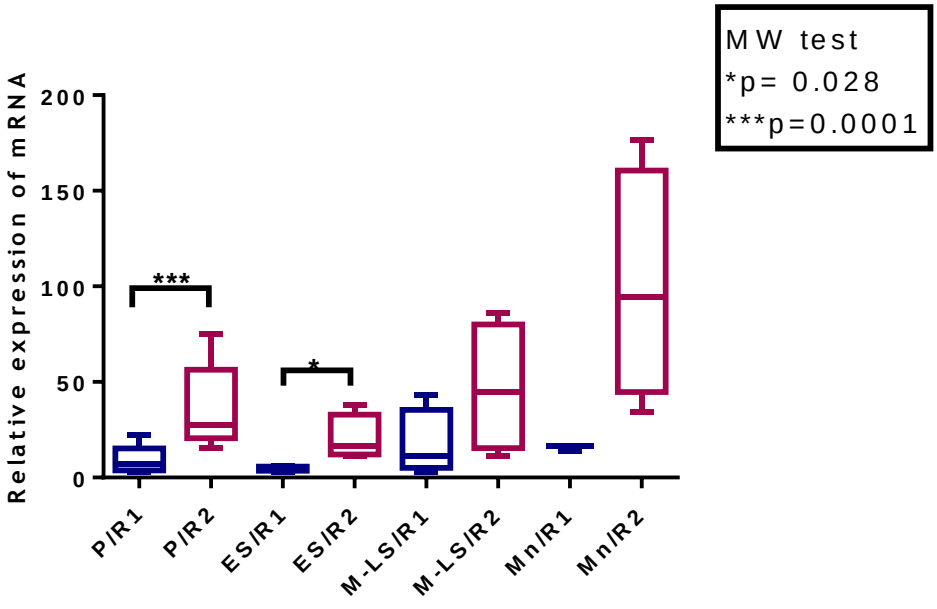


Figure 5.11 : The differences in gene expression for VEGF-R1 and VEGF-R2 across the menstrual cycle; in proliferative (P), early secretory (ES), mid to late secretory (M-LS) and menstrual (Mn). * for P ≤0.0001,* for P<0.05. The actual values of the relative expression of mRNA were analysed by Mann Witney test.**

5.3.4 Expression of VEGA in uterine tissue in women with fibroids

5.3.4.1 Changes in VEGF-A mRNA level across the menstrual cycle in endometrium

The result of RT-PCR data about endometrial VEGF-A in women with uterine fibroids is shown above (section 5.3.2.2).

5.3.4.2 Changes in VEGF-A mRNA level across the menstrual cycle in myometrium

RT-PCR data analysis shows that the expression of VEGF-A was higher in the proliferative phase than the secretory phase (P=0.035) (Figure 5.112). However, by excluding the outlier, the difference between the two phases was not significant (0.057).

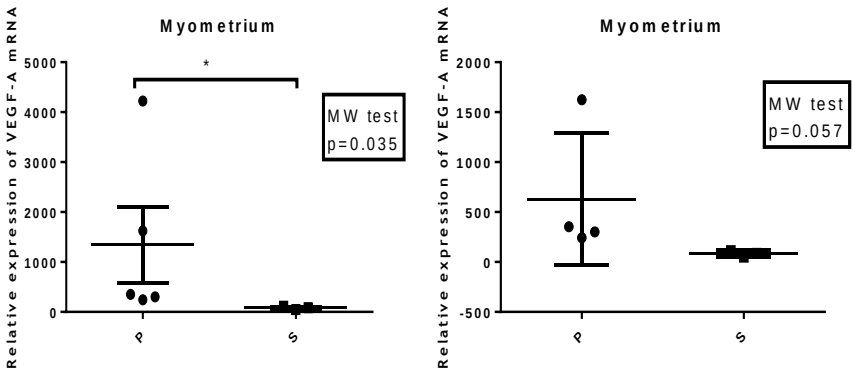


Figure 5.12: The expression of VEGF-A mRNA in myometrium in women with uterine fibroids during proliferative (P) and secretory (S) phases. The actual values of the relative expression of VEGF-A mRNA were analysed by Mann Witney test.

5.3.4.3 Changes in VEGF-A mRNA level across the menstrual cycle in fibroid

There was no difference in the expression of VEGF-A mRNA level between the proliferative and secretory phase in fibroids tissue (Figure 5.13).

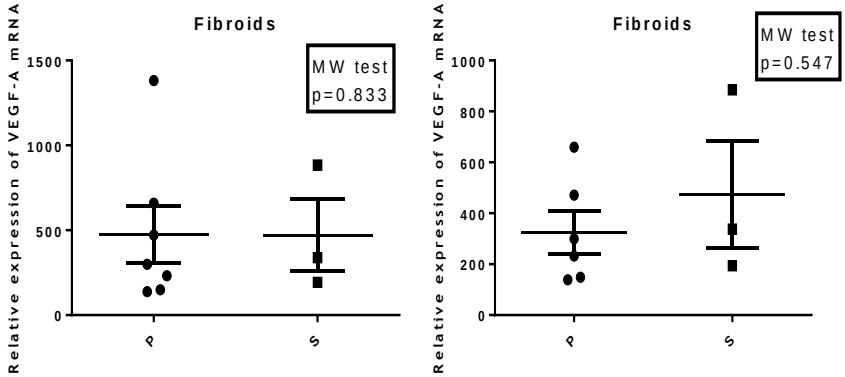


Figure 5.13: The expression of VEGF-A mRNA in fibroid tissue in the proliferative (P) and secretory (S) phases. Statistical analysis with and without outlier was done for the actual values of the relative expression of VEGF-A mRNA by Mann Witney test.

5.3.4.4 Difference in the expression of VEGF-A protein between uterine tissue

In addition to endometrium (section 5.3.1.2), shows wide infiltration of VEGF-A in both myometrium and fibroid tissue for women with different types of uterine

fibroids. Immunostaining intensity of VEGF-A protein was higher in the endometrium and fibroids tissue than the adjacent myometrium tissue (Figure 5.14).

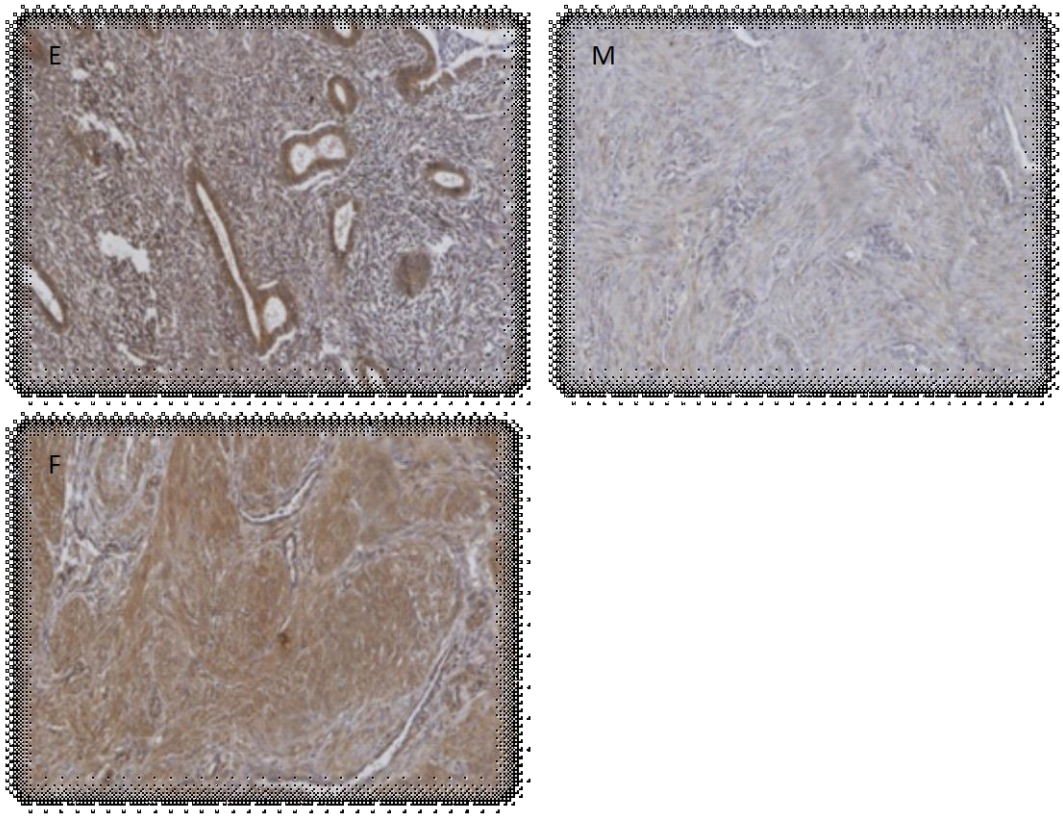


Figure 5.14: Representative immunostaining tissue for uterine fibroid tissue of women had uterine fibroids, shows the expression of VEGF-A in endometrium (E) myometrium (M) and fibroid (F)

5.3.4.5 Difference in the expression of VEGF-A mRNA between uterine tissue

RTPCR data analysis shows no difference in VEGF-A mRNA level in endometrium, myometrium and uterine fibroids (p= 0.375) (Figure 5.15).

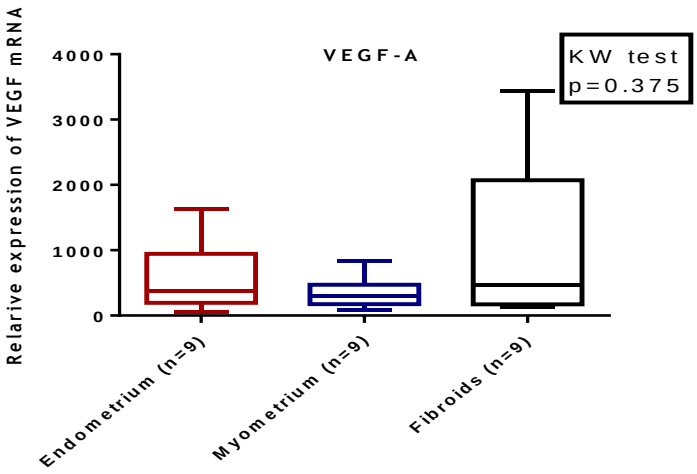


Figure 5.15: The expression of VEGF-A mRNA in uterine tissues and uterine fibroids in women with HMB. The actual values of the relative expression of VEGF-A mRNA were analysed by Kruskal Wallis test.

5.3.4.6 Difference in the expression of VEGF receptors mRNA level in uterine tissue

There was no significant variation in the two receptors (VEGF-R1 & VEGF-R2) mRNA expression between endometrium, myometrium and different type of uterine fibroids (p= 0.649&0.958 respectively) (Figure 5.16).

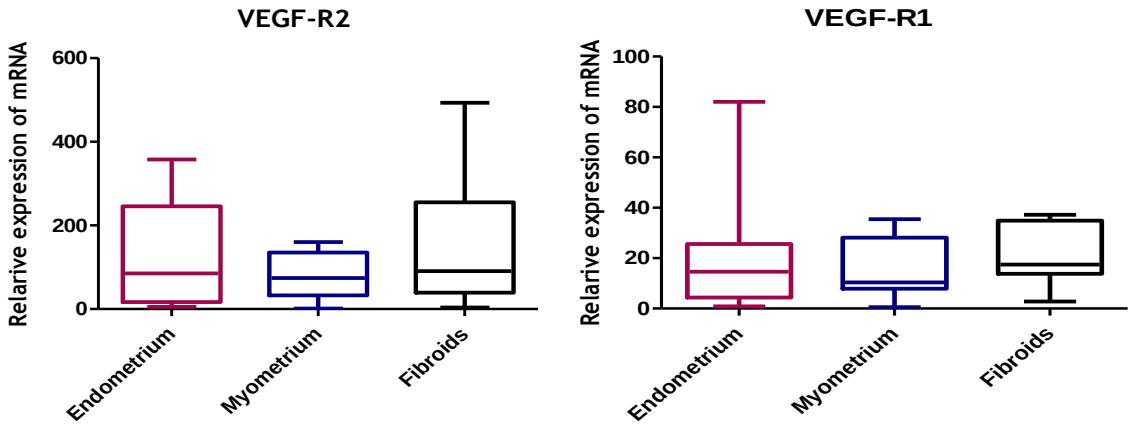


Figure 5.16: Box plots show the differences in the relative expression of VEGF-R1 mRNA between uterine fibroid tissue; endometrium, myometrium and fibroid.

5.3.4.7 The expression of VEGF-A mRNA in women treated with GnRH agonists

RT-PCR results for VEGF-A mRNA shows higher expression in the endometrial VEGF-A in women had GnRH agonists than that for women had no hormonal treatment (P= 0.007) (Figure 5.16).

There was no difference in the expression of VEGF-A mRNA in fibroids tissue for women had GnRH and women had no hormonal treatment (P= 0.050) (Figure 5.17).

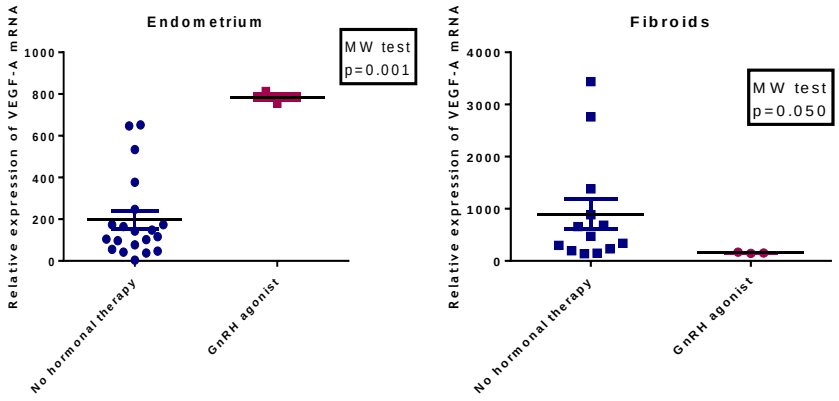


Figure 5.17: The difference in the expression of VEGF-A mRNA in endometrium and fibroid tissue for women with uterine fibroids who had GnRH agonists and in women had no hormonal therapy.

5.3.5 VEGF-A serum levels across the menstrual cycle in women with HMB

5.3.5.1 In women with normal uteri

The experimental results of ELISA technique show no difference in VEGF serum level across the menstrual cycle for women had idiopathic HMB (Figure 5.18).

5.3.5.2 In women with uterine fibroids

No difference in the VEGF serum level across the menstrual cycle for women with uterine fibroids as well (Figure 5.18).

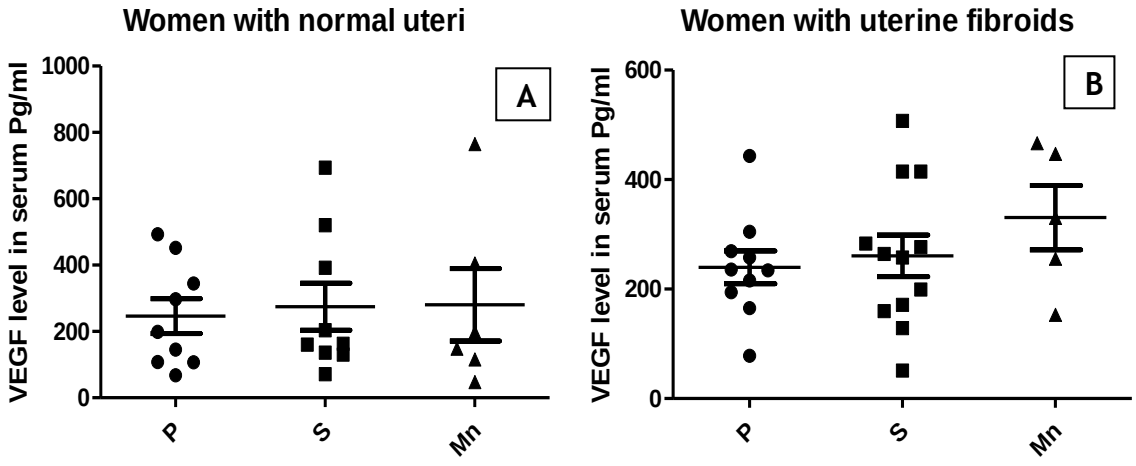


Figure 5.18: Serum levels of VEGF in women had HMB with normal uteri (A) and with uterine fibroids (B). The actual values were analysed by kruskal wallis test and show no difference in the serum levels of VEGF across the menstrual cycle.

5.3.5.3 Differences in VEGF serum level between groups

There was no statistical difference in the concentration of serum VEGF between women with normal uteri and women with uterine fibroids in case of HMB (Table 5.4).

Table 5.4: Differences in VEGF serum level between women with uterine fibroids and women with normal uteri, both had HMB.

	VEGF concentration in serum (pg/ml) (range)			
	In women with fibroid uterus		In women normal uteri	
menstrual	N= 5	330.7(153.4-466.8)	N=6	280.3(48.29-765.65)
Proliferative	N=10	443.1(78.21-443.1)	N=9	246(68.25-492.6)
Early secretory	N=8	292.1(128.9-507.0)	N=4	362.4(160.4-693.7)
Mid-late secretory	N=4	197.8(51.41-283.1)	N=5	204.2(71.36-520)
In GnRH	N=3	413.12 (185.13-575.9)		

5.3.5.4 Relation between VEGF concentration in serum and estradiol level in women with HMB

There was no correlation between the VEGF serum concentration and estradiol level in blood in women with normal uteri and women with uterine fibroids (Figure 5.19&5.120).

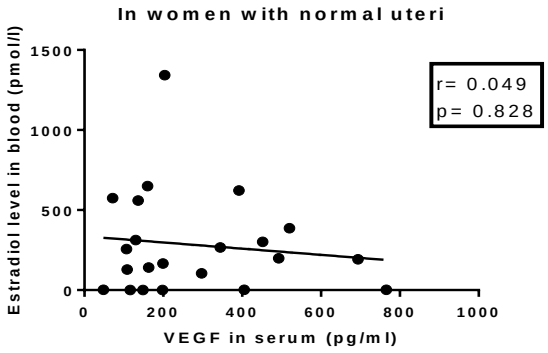


Figure 5.19: Correlation between the VEGF serum concentration and estradiol level in blood for women with no uterine pathology. Spearman correlation test was used.

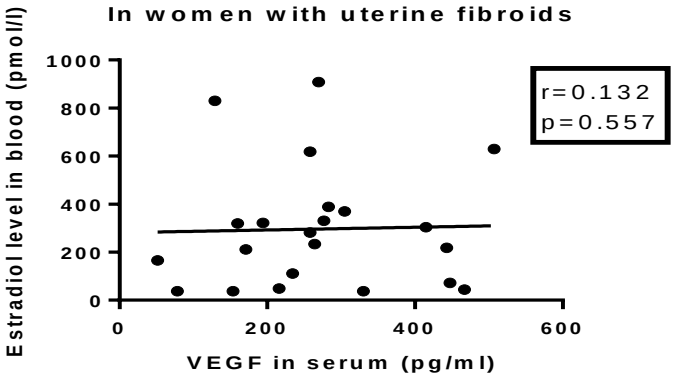


Figure 5.20: Correlation between the VEGF serum concentration and estradiol level in blood for women with uterine fibroids was not statistically significant. Spearman correlation test was used.

5.4 Discussion

In this study, the data presented a consistent relationship between the RT-PCR results and IHC results regarding the endometrial VEGF-A expression across the menstrual cycle for women with HMB and uterine fibroids compared with women who had idiopathic HMB.

Although the endometrial VEGF-A increased across the menstrual cycle, it increased mainly during the menstruation in women with idiopathic HMB. However, this pattern was also obvious in these with HMB associated with uterine fibroids, where the endometrial VEGF-A increased mainly during menstruation. Previous studies about the expression of endometrial VEGF-A presented conflict results. Some of these reports highlighted high expression of VEGF-A in the secretory phase [320],while others pointed to high expression in the proliferative phase [94, 325, 326]and some of authors presented increased expression in the peri and post menstruation [313, 315].

Almost all the previous studies agreed about the role of VEGF in neoangiogenesis. Endometrial regeneration within the remaining functionalis and the basalis layers and neoangiogenesis process occur simultaneously and shortly after starting the menstrual bleeding. This process is thought to be triggered by regional hypoxia. VEGF-A is one of the genes that have hypoxia response elements in its promoter and it is induced by hypoxia in many cells [360-362]. Therefore, VEFG-A is discovered in the endometrial stroma in the late secretory and menstrual phases (decidualised stroma) and in the menstrual and early proliferative phases (non- decidualised stroma), hence, in peri and post menstruation endometrial stroma can respond to hypoxia by augmented production of VEGF-A. The expression of VEGF-A in stromal and glandular endometrium during the secretory and proliferative phases also is under the regulation of estradiol and progesterone as well as the hypoxia and some evidence about the role of cyclic AMP (cAMP) as an independent regulatory pathway. In that, disturbances in these regulatory factors can lead to alternation in the VEGF gene expression in endometrium and cause menstrual abnormality.

In this study , we could not find a clear difference in endometrial VEGF-A production between women who had idiopathic HMB and women who had HMB in the presence of uterine fibroids, although, the IHC data identified an increase in the endometrial VEGF-A during the early secretory phase in women with

uterine fibroids greater than that in women with normal uteri. On the other hand, this finding was not consistent with the RP-PCR data, which identified no difference in the endometrial VEGF-A production between the two groups across the menstrual cycle. These results suggest that endometrial VEGF-A plays the same role in heavy menstrual bleeding in women with uterine fibroids as that in idiopathic heavy menstrual bleeding.

The present study also showed considerable endometrial expression of KDR (VEGF-R2) and flt-1 (VEGF-R1) mRNAs across the menstrual cycle in women with HMB, both KDR and flt-1 show highest expression in the menstrual phase. This finding is consistent with a report by Graubert et al. [315] and Nayak et al[363], who demonstrated high immunohistochmical KDR expression in premenstrual phase. In contrast, other RT-PCR analysis showed that KDR mRNA in isolated endometrial stroma was increased during the secretory phase [306]. However, other study found no variation in KDR and flt-1 mRNA values throughout the menstrual cycle [364].

The majority of studies that examined KDR and flt-1 in endometrium, concentrated on their expression in stromal and epithelial glandular cells. Some of these studies observed immunostaning infiltration of both KDR and flt-1 in the stromal and glandular endometrial tissue [365, 366],and other studies showed no significance of immunostaining for flt-1 compared with KDR[306]. The present study found a higher expression of KDR mRNA than flt-1mRNA during the proliferative, and menstrual phase, and this is in one part consistent with a result by Meduri et al. [365], where KDR was higher than flt-1 at the beginning and during the proliferative phase in women with normal menstrual cycles. In contrast, Mints et al. [314] found higher immunostaning expression of flt-1 than KDR particularly during the secretory phase. However, this finding was regarding VEGF receptors in endometrial blood vessels in women with HMB. Thus, the results on flt-1 and KDR expression in the human endometrium are still controversial. This variation may be due to differences in the experimental technique, sample preparation, and probes or antibodies used.

The study showed similar pattern of expression of flt-1 across the menstrual cycle to that of VEGF-A in women with idiopathic HMB. This finding may indicate similar regularity factors. This suggestion is consistent with a report by Gerber

et al.[367] who indicated that unlike KDR, both VEGF and flt-1 are directly up regulated by hypoxia via hypoxia inducible factor -1 as both of them have a HIF-1 consensus in their promoter region. Therefore, this boosts the enquiry whether flt-I might contribute to a modulated biological effect of VEGF-A in HMB patients. Although VEGA binds to flt-1 in high affinity [368], its cellular effect appears to be more predominantly mediated by KDR in endothelial cells [369-371].However, our results found no correlation between the level of VEGF-A mRNA and KDR and flt-1 mRNAs, except in the proliferative phase where the increase in the VEGF-R2 corresponded to the production of endometrial VEGF-A. In addition, the study showed increase in flt-1 during the mid to late secretory phase. Therefore, this may indicate strong kinase activity of KDR in response to VEGF-A during the proliferative phase. Additionally, the negative regulatory action of flt-1 on angiogenesis [372, 373] and the mediatory action of flt-1 for endothelial cell migration [374, 375] mainly occurs during the mid-secretory phase in cases of HMB.

The expression of VEGF-A protein showed stronger immunostaining intensity in uterine fibroids than in adjacent myometrium. This is consistent with a report by Gentry et al.[376]. However, this finding was not supported by our RT-PCR data where there was no difference in the VEGF-A mRNA level between uterine fibroids and myometrium and this concurs with Harrison-Woolrych et al. 1995[356] . Furthermore, the level of KDR and flt-1 mRNAs was insignificantly higher in uterine fibroids than myometrium[377]. Nevertheless, collected myometrium and uterine fibroid samples for RT-PCR were not in similar adjacent manner. In contrast, another study found higher level of VEGF mRNA expression in women with uterine fibroids than women with healthy myometrium[378]. Nonetheless, the later study investigated uterine fibroids and myometrium from different subjects and it had the advantage of bigger sample size. Our study suggests that the high expression of VEGF-A in uterine fibroids indicates that local angiogenesis may be important for the development and growth of this tumour. In addition, our findings suggest that increase level of VEGF-A mRNA expression in myometrium in women with multiple uterine fibroids compared with expression of VEGF-A in case of single uterine fibroids as most our subject had multiple uterine fibroids. Hence, VEGF-A may have a role in the development and growth of uterine fibroids. Furthermore, development and growth of uterine fibroids may up regulate the VEGF production in myometrium

and cause development of further fibroids. However, VEGF is not mitogenic to smooth muscle cells, but is a principal mediator of angiogenesis[356].

In this study, the collected tissue were clinical samples, which obtained mainly for clinical diagnosis or treatment and this to some extent constrained having enough fresh samples for RT-PCR, so RNA was extracted from paraffin samples. However, the quality of RNA extracted from paraffin was quantified and compared with that extracted from frozen samples, but still a single RNA extraction method is preferred. Furthermore, the same reason constrains having larger sample size for all stages across the menstrual cycle. Nonetheless, the sample size was in similar to these of other studies investigating the same markers [94,215,314,365,379]. In our study, there was no excluding for confounding factors such as smoking and dysmenorrhea, which may affect the result with small sample size. Furthermore, if the expression of VEGF-A is different during the early and late proliferative phase, staging the proliferative phase would pick up more of the variation in expression of VEGF-A between proliferative and secretory phase and would validate more the results. However, many studies in the research field have not had staging the proliferative phase as well [215, 380].
GnRH-agonists are an effective therapy for a wide number of gynaecological disorders. In women with uterine fibroids, the administration of GnRH-agonists is associated with a significant decrease in uterine and fibroids volume and with an improvement in fibroids-related symptoms. These effects are reversible at the end of the therapy that is therefore considered as a preoperative treatment.

Our data shows a positive immunoreactivity for VEGF-A in endometrium, myometrium and uterine fibroids in women preoperatively treated with GnRH agonists. In endometrium, there was a reduction in VEGF-A protein expression in stromal and epithelial cells after therapy, but the remaining stained cells maintained a marked immunostaining. A compensatory reaction could be a possible explanation like that seen in physiological condition. The effect of GnRH-agonist on the expression of VEGF protein in myometrium and uterine fibroids was similar pattern of endometrium, and it was identified in both smooth muscle cells and fibroblast. However, the expression of VEGF protein was strong in endothelial cells in treated uterine fibroids, and this consistent

with other work[381]. The persisting vascular reservoir of VEGF could be among the possible explanations of the rapid regrowth of uterine fibroids after GnRH-a therapy is stopped. Although the VEGF-A mRNA expression in myometrium and uterine fibroids showed no difference between treated and non-treated group. In endometrium, our data identified higher expression of VEGF-A in treated group than non-treated group. The effect of GnRH agonists of VEGF is controversial, while some authors found decrease in the VEGF level in plasma and follicular fluid in women treated with GnHR agonist[382-384], other reports demonstrated increase in its level. However, some studies found no difference in its effect on EGF expression [385].nevertheless, hetrogencicty in used methodology may contribute this diversity.

5.5 Conclusion

In conclusion, the VEGF-A expression is higher during the menstrual phase than proliferative and secretory phases. It seems that VEGF-A is involved in heavy menstrual bleeding mechanism in women with uterine fibroids similar to these with women with idiopathic HMB. VEGF-A has been suggested to have a role in the development and growth of uterine fibroids, however, uterine fibroids may up regulate production in myometrium and cause further development of uterine fibroids.

Additional investigations are required to identify the pattern of VEGF expression in case of HMB with larger sample size than that used in previous studies. Further investigations are needed to identify more about the signal pathway of flt-1 and its main role in mediating the VEGF-A function. Detailed research about the VEGF expression in myometrium for women with uterine fibroids compared with women with normal uteri may help to understand the pathophysiology of uterine fibroids.

Chapter 6

6. Prostaglandin E2 in heavy menstrual bleeding and in presence of uterine fibroids

6.1 Introduction

Prostaglandins (PGs) are part of the eicosanoid family and consist of five endogenous members, called prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), prostaglandin F2α (PGF2α), prostacyclin, and thromboxane A2. The prostaglandins are bioactive metabolites, derived from the action of cyclo-oxygenase (COX) enzymes upon arachidonic acid (AA). In summary, cyclooxygenase enzymes (COX-1& COX-2) generate PGG2 from arachidonic acid, the former reduced to the unsTable intermediate (PGH2) from which other prostanoids derived by range of prostaniod enzymes and isomerase (Figure 6.1). COX-1 is a constitutive enzyme expressed in nearly all body tissues and cell types and generates PG for normal physiological function. However, previous studies have shown upregulation of COX-1 expression in carcinomas[386].Pro-angiogenic factors can rapidly induce COX-2 in cells such as growth factors, oncogenes and carcinogens and various types of stimuli (). A role has been identified for COX-2 in different pathological conditions such as rheumatic diseases, inflammation and tumorigenesis [387]. Prostaglandin E2 is a vasodilator and works via four pharmacological classified subtypes of G protein- coupled receptor (EP1-EP4)[388].These subtypes may be coexpressed on the same cell or on adjacent cells, indicating an autocrine/paracrine control of an autocoid biosynthesised and released in close proximity to the site of its action[389].

Prostanoids work mainly as primary mediators in pathological conditions such as inflammation, hypertension and cancer but they also have a crucial role in normal physiological function such as in the female reproductive system[390]. This concept was supported by observational studies which investigated the effect of COX-1 and COX-2 deficient mice. These observations confirmed that COX-1 has an essential role for determining the normal gestational period and parturition [391, 392]. Reduction in COX-2 has detrimental effects on ovulation, fertilization, implantation and decidualization, and this is an example of the role of prostaglandins produced by COX-2 in these processes [393-396]. COX-2 expression and PGE2 synthesis is upregulated by pituitary gonadotropin before ovulation and this promotes follicle expansion and ovulation. Hence,

administration of exogenous PGE2 has been shown to save ovulation in mice [394]. In human reproduction, dysregulation in endometrial prostanoids can cause menstrual disorder [79,397,398].

In the normal menstrual cycle, cyclical tissue breakdown and remodelling occurs in human endometrium. This process is mainly under the influence of sex steroid hormones. Towards the end of menstrual cycle and parallel with the withdrawal of progesterone and sloughing of the functional layer of endometrium, COX-2 expression is upregulated in human endometrium [269, 386, 399], with subsequent synthesis of PGE2 and PGF2α (a vasoconstrictor)[400]. This occurs at the same time as an increase in the expression of prostaglandin E2 receptors (EP2)[401]. In addition, COX-2 induced prostaglandins regulate leukocyte influx from the circulation to endometrium, and this leads to endometrial breakdown [402, 403]. Recent studies have determined a role for COX enzymes and PGS in the regulation of epithelial cell growth and angiogenesis[404]. In addition, overexpression of COX-2 and PGE2 synthesis are associated with increased cellular proliferation and apoptosis resistance [405]. Furthermore, they have a direct effect on the endothelial cell function[406] and reflect their association with vascular function . Hence, treatment of endothelial cells with selective COX-2 inhibitors has been shown to reduce microvascular tube formation [404, 407]. In addition, some studies performed in cancer cell lines suggested that PGE2 act via EP2 to generate VEGF expression through HIF-1 activation [62, 408]. Hence, it is assumed that there are possibly two pathways connecting COX-2 up-regulation with downstream angiogenic gene expression; increased PGF2 expression leading to local ischemia/hypoxia and thus HIF-1 expression, and increased PGE2 expression directly inducing HIF-1expression[409].

In women suffering from heavy menstrual bleeding, excessive expression of COX-2 and PGE2 synthesis in endometrium has been recognised[410]. In this group of women, a positive relationship between the volume of blood loss and PGE2 release in the uterus has been identified [96].

The aim of this chapter is to study the expression of COX-2 and the PGE2 synthesis in human endometrium in heavy menstrual bleeding and the difference in presence of uterine fibroids.

6.2 Methods

Materials and methods were discussed in chapter two.

6.3 Results

6.3.1 COX mRNA and protein expression within human endometrium in heavy menstrual bleeding across the menstrual cycle

6.3.1.1 In women with idiopathic HMB

The expression of COX enzyme mRNA in human endometrium in heavy menstrual bleeding was determined by Taqman quantitative RT-PCR analysis. For COX-1 mRNA, no difference in the gene expression was identified across the menstrual cycle (Figure 6.1).

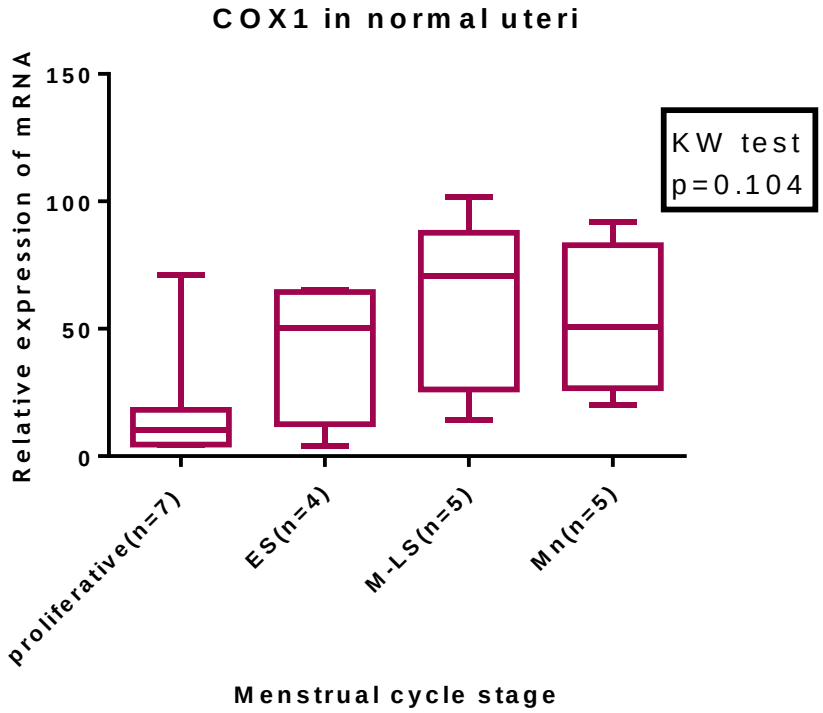


Figure 6.1: The expression of COX-1 mRNA in endometrium in women with idiopathic heavy menstrual bleeding. Proliferative (P), early secretory (ES), mid- late secretory (M-LS), and menstrual (Mn) phases. The actual values of the relative expression of COX1 mRNA were analysed using Kruskal Wallis and show no difference in the expression of COX-1 mRNA across the menstrual cycle.

Significant differences in the expression were noted for COX-2 between menstrual and proliferative phase (median of 0.78 (0.3, 4.7) and 31.7 (3.55, 230) respectively, P<0.05) (Figure 6.2). In addition, the immunostaining analysis shows that COX-2 protein was highly expressed in the endometrial epithelial and endothelial cells with no change across the menstrual cycle (Figure 6.3). However, COX-2 protein expression in endometrial stroma was higher during the mid-secretory than the menstrual phase (2.3±0.3, 1.2±0.2 respectively, p<0.05) (Figure 6.4& 6.5).

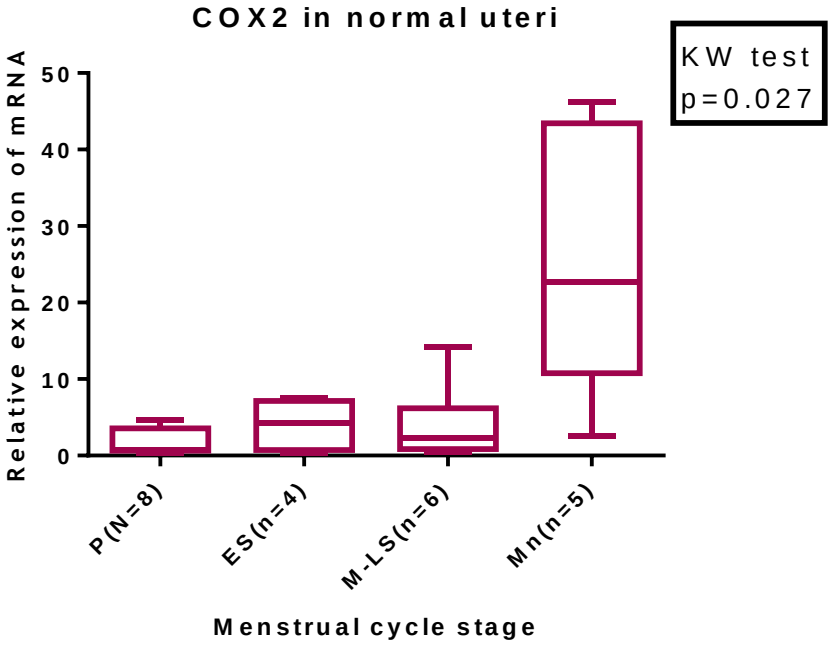


Figure 6.2: The expression of COX-2 mRNA in endometrium in women with idiopathic heavy menstrual bleeding.Proliferative (P), early secretory (ES), mid- late secretory (M-LS), and menstrual (Mn) phases. The actual values of the relative expression of COX2 mRNA were analysed using Kruskal Wallis test.

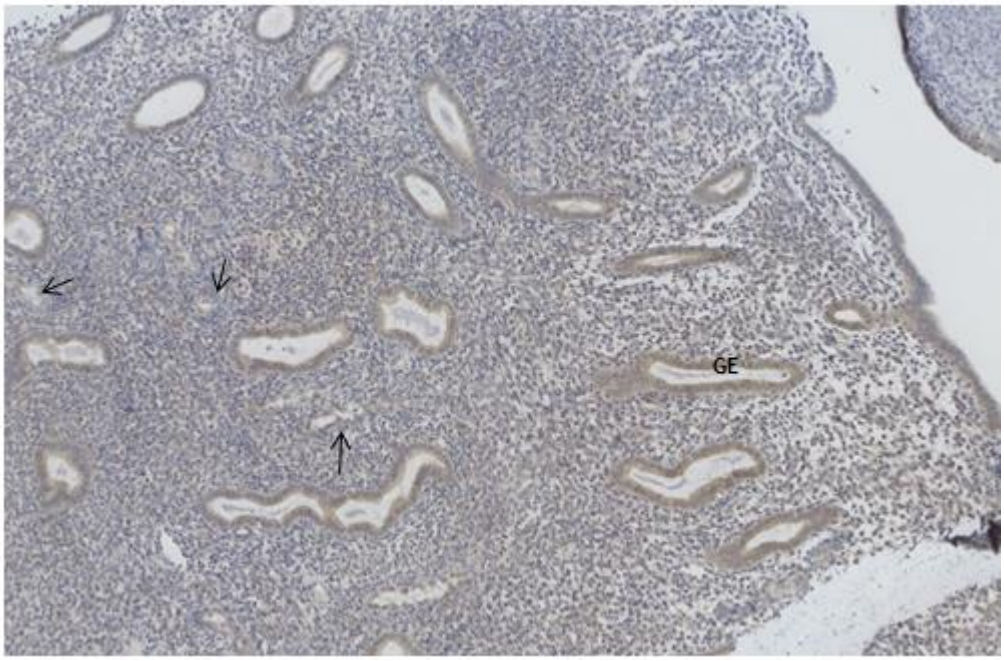


Figure 6.3: The expression of COX-2 protein in glandular epithelial cells (GE) and endothelial cells (arrows). The brown stained cells are positive for COX2 protein and other cells in blue colour show no COX2 protein expression.

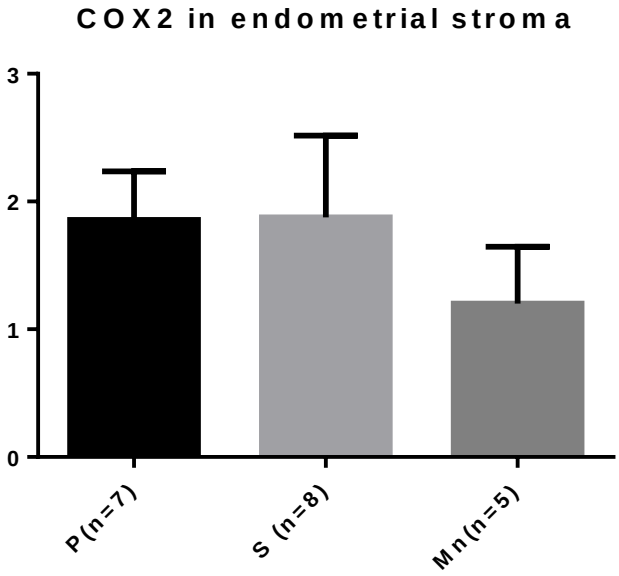


Figure 6.4: Immunostaining intensity of COX-2 protein expression in endometrial stroma (S) and gepithelial glands (G) in women with idiopathic heavy menstrual bleeding during proliferative (P), secretory (S) and menstrual (M) phases. Semiquantitative 4 scale scoring method was used, 1 for mild (<10%), 2 for moderate (10-50%) and 3 for intense staining (>50%).

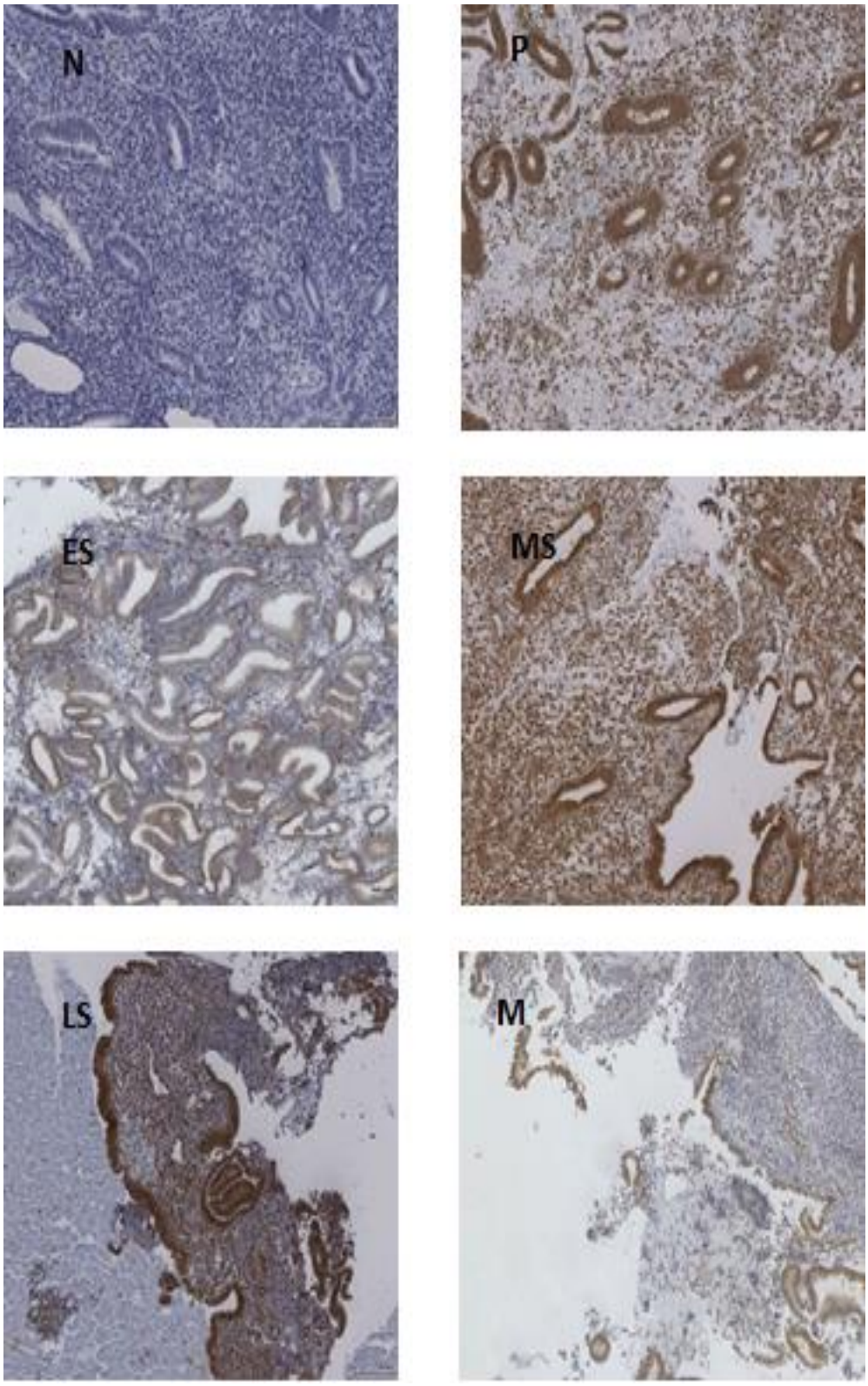


Figure 6.5: COX-2 protein expression in endometrium across the menstrual cycle for women with idiopathic heavy menstrual bleeding; negative control (N), proliferative (P), early secretory (ES), mid-secretory (MS), late secretory (LS) and menstrual (M) phases. The cells in brown immunostaining are positive for COX2 protein and other cells in blue colour show no COX2 protein expression.

6.3.1.2 In women with uterine fibroids

In women with heavy menstrual bleeding and uterine fibroids, there were no significant differences in the expression of endometrial COX-1 across the menstrual cycle at the mRNA level (Table 6.1). In addition, the variation in COX-2 gene expression (Figure 6.6) and intensity score of COX-2 protein expression (Figure6.7) was not significantly difference. On the other hand, COX-2 protein expression was reduced in stromal cells from the functional and the basal layer of the endometrium with no change for glandular epithelial and endothelial cells (Figure 6.8).

Table 6.1: The expression of COX-1 protein in endometrium of women with uterine fibroids

	The relative expression of COX-1 mRNA (median (range))
Menstrual	50.66 (1.4-144.5)
Proliferative	10.21 (4.4-71.4)
Early secretory	100.2 (3.9-165.4)
Mid-late secretory	70.8 (14.2-173.6)

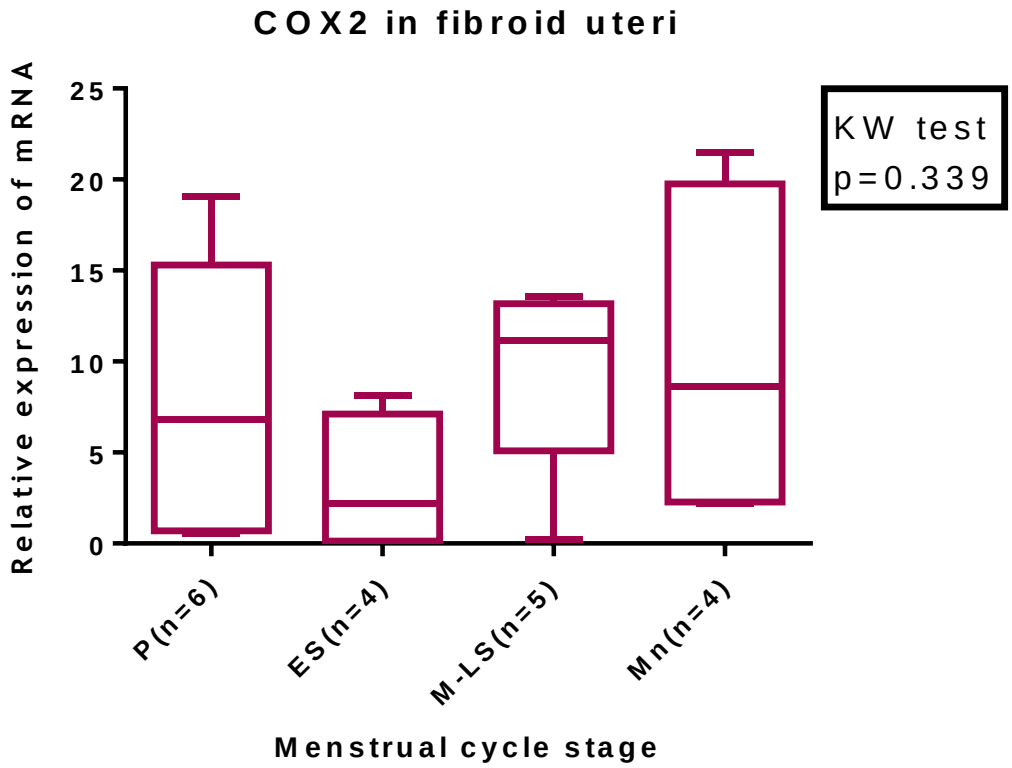


Figure 6.6: The expression of COX-2 mRNA across the menstrual cycle in women with HMB and uterine fibroids. The actual values of the relative expression of COX2 mRNA were analysed using Kruskal Wallis test.

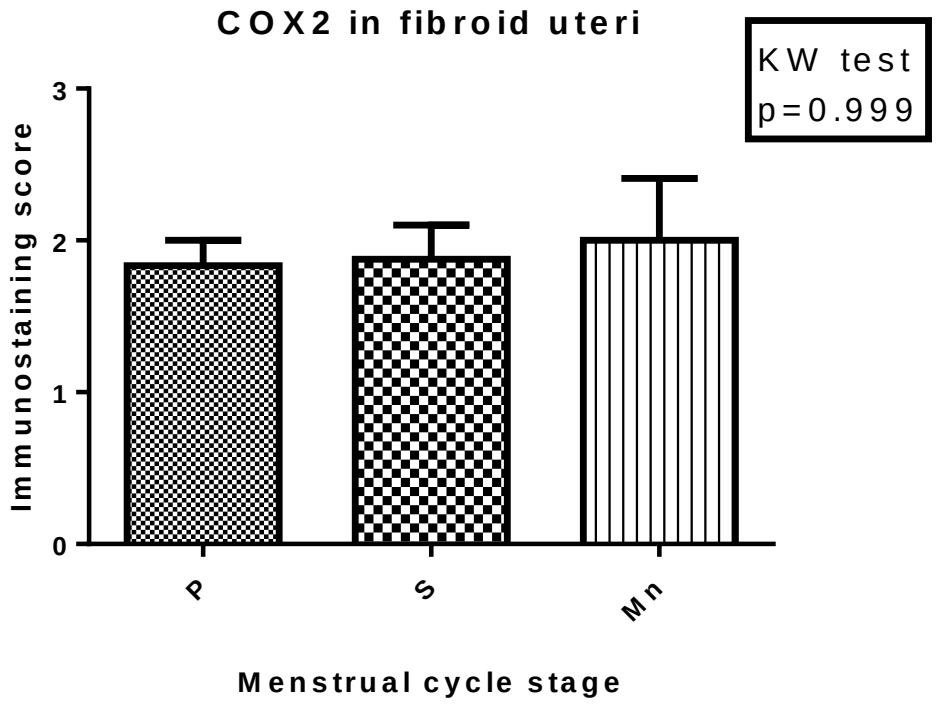


Figure 6.7: Immunostaining score of COX-2 endometrial stroma in women with uterine fibroids. Semiquantitative 4 scale scoring method was used, 1 for mild (<10%), 2 for moderate (10-50%) and 3 for intense staining (>50%).

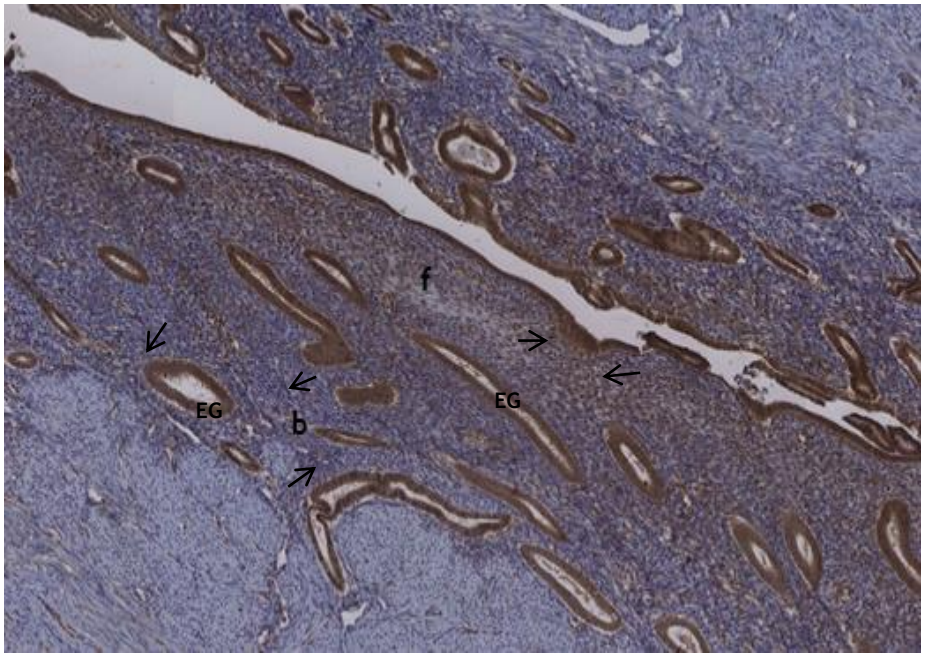


Figure 6.8: expression of COX-2 protein in stromal cells (arrows) in functional (f) was higher than that in basal layer (b) of endometrium in women with heavy menstrual bleeding and uterine fibroids while no difference in COX-2 expression in epithelial glandular cells (EG) between the functional and the basal layer of endometrium. The cells in brown immunostaining are positive for COX2 protein and other cells in blue colour show no COX2 protein expression.

6.3.2 **COX-2 protein expression in myometrium and fibroid tissue in women with uterine fibroids**

In myometrium, the expression of COX-2 protein was mainly in perivascular cells (Figure 6.9). In uterine fibroids, there was wide infiltration of COX-2 protein (Figure 6.10).

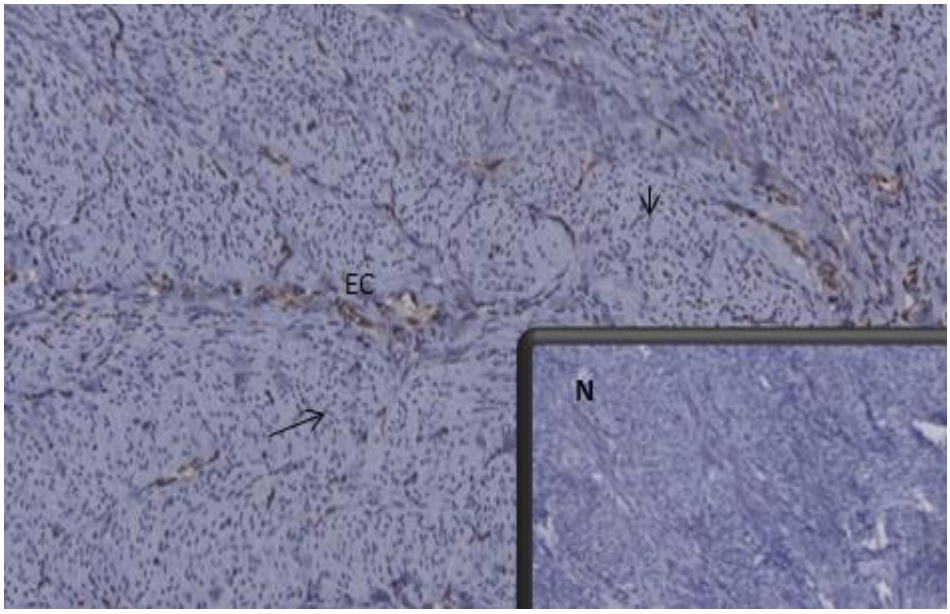


Figure 6.9: COX-2 protein expression in myometrium in women with uterine fibroids and heavy menstrual bleeding; endothelial cells (EC), stromal cells (arrows) and negative control myometrium section (N). The cells in brown immunostaining are positive for COX2 protein and other cells in blue colour show no COX2 protein expression.

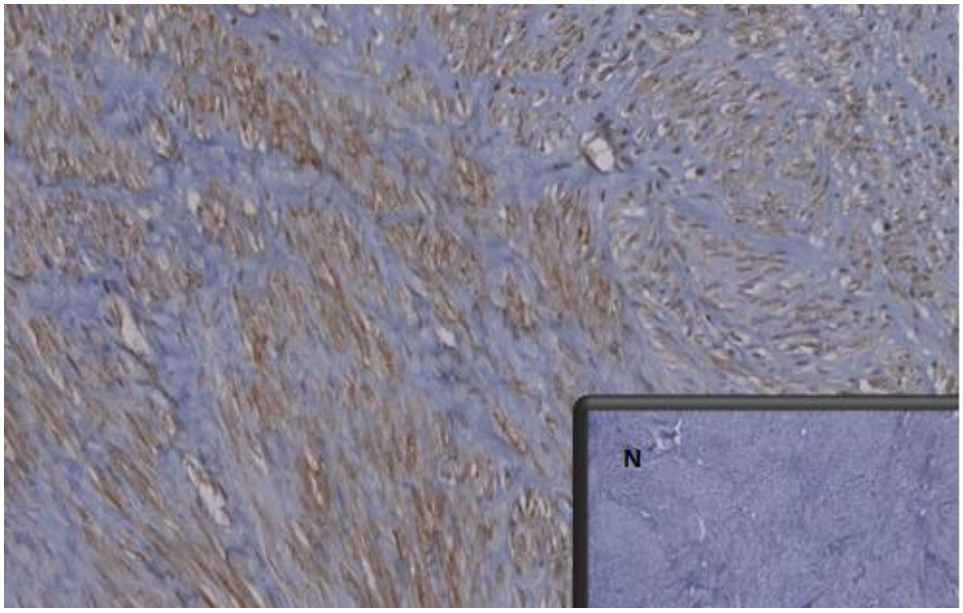


Figure 6.10: COX-2 protein expression in fibroid tissue in women with heavy menstrual bleeding. Negative control fibroid section (N). The cells in brown immunostaining are positive for COX2 protein and other cells in blue colour show no COX2 protein expression.

6.3.3 PGE2 and EP2 expression in endometrium in heavy menstrual bleeding.

6.3.3.1 In women with idiopathic HMB

Immunohistochemistry was used to identify the expression of PGE2 in endometrium and the results show no difference in PGE2 protein infiltration across the menstrual cycle (Figure 6.11& 6.12). Furthermore, the PGE2 protein expression was high in both epithelial glandular and stromal endometrial cells. By using the RT-PCR technique, the expression of EP2 mRNA, was not significantly various between phases throughout the menstrual cycle (Figure 6.13).

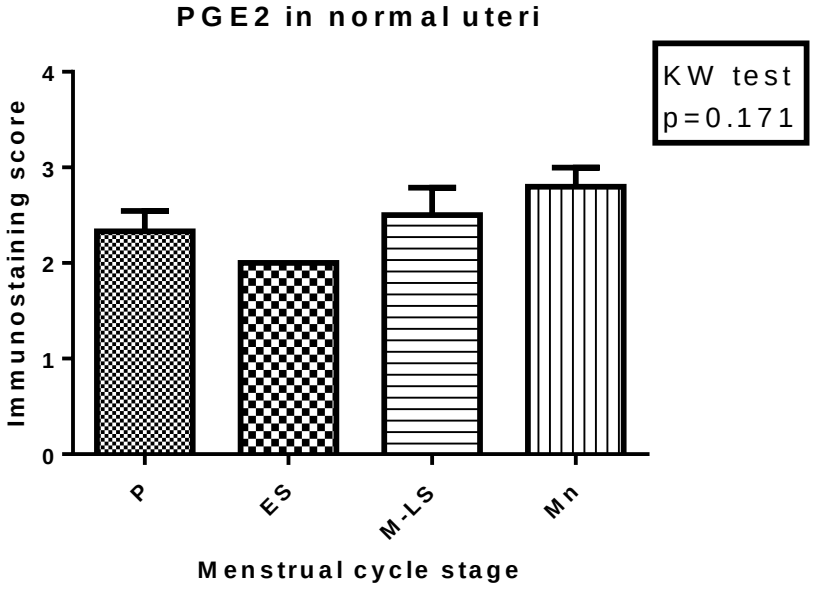


Figure 6.11: Imunostaining intensity level of PGE2 protein in endometrium in women with idiopathic heavy menstrual bleeding across the menstrual cycle. Semiquantitative 4 scale scoring method was used, 1 for mild (<10%), 2 for moderate (10-50%) and 3 for intense staining (>50%).

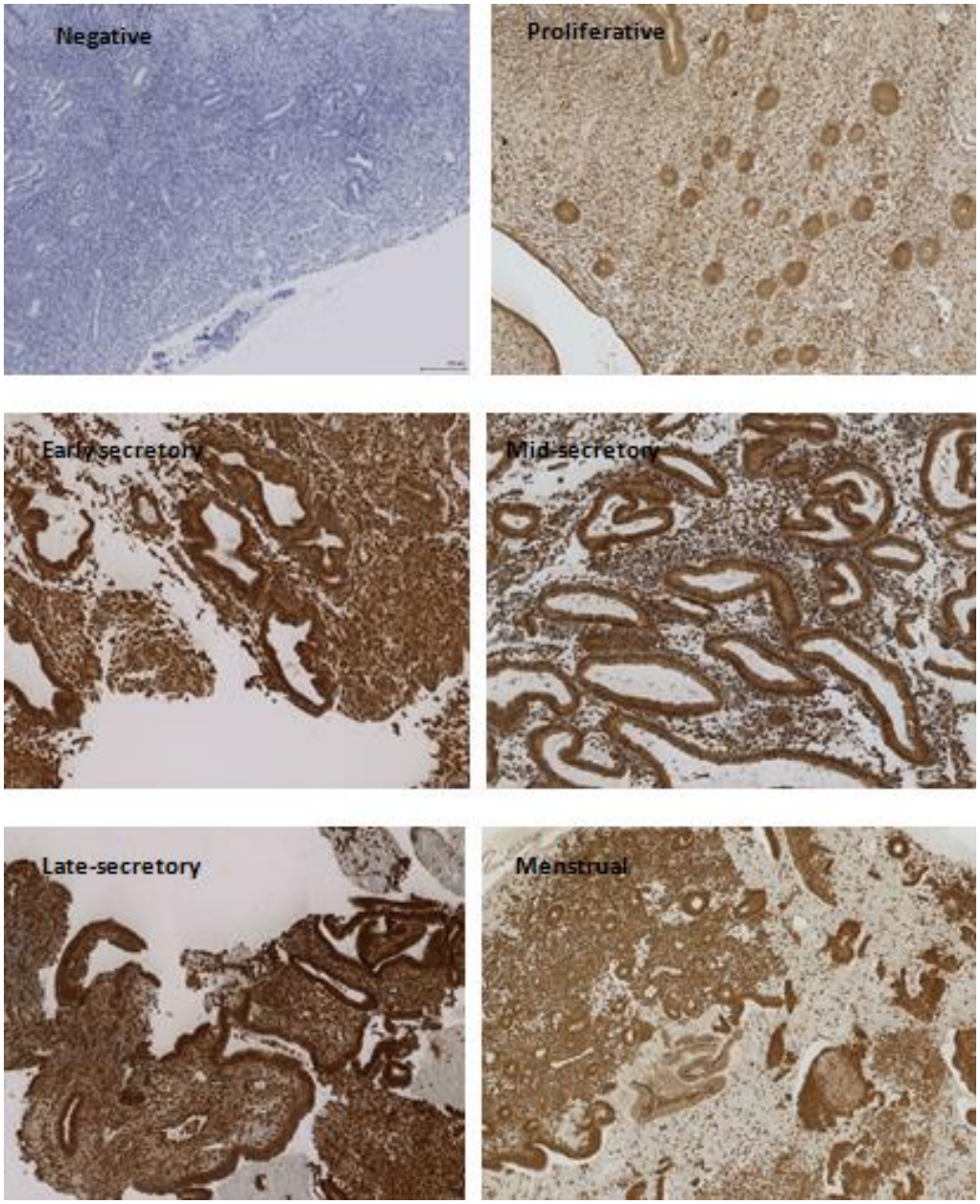


Figure 6.12: High expression of PGE2 in endometrium for women with heavy menstrual bleeding throughout the menstrual cycle in both stromal and glandular endometrial tissue. The cells in brown immunostaining are positive for PGE2 protein and other cells in blue colour show no PGE2 protein expression.

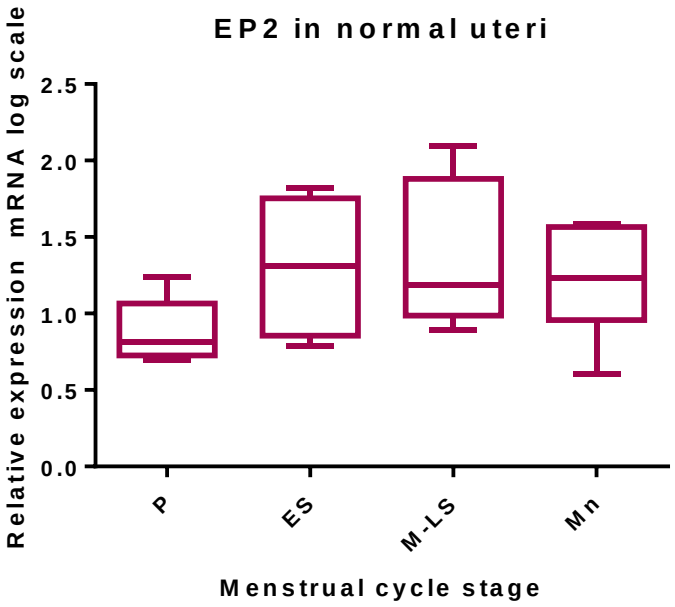


Figure 6.13: The expression of EP2 mRNA in endometrium for women with idiopathic heavy menstrual bleeding across the menstrual cycle. (Log scale was depicted).

6.3.3.2 In women with uterine fibroids

In full thickness endometrium collected from women with uterine fibroids, PGE2 protein expressed in both basalis and functionalis layer with reduction in the stromal compartment in the basalis layer. However, no change was noticed for epithelial, endothelial and intraglandular infiltration (Figure 6.14).

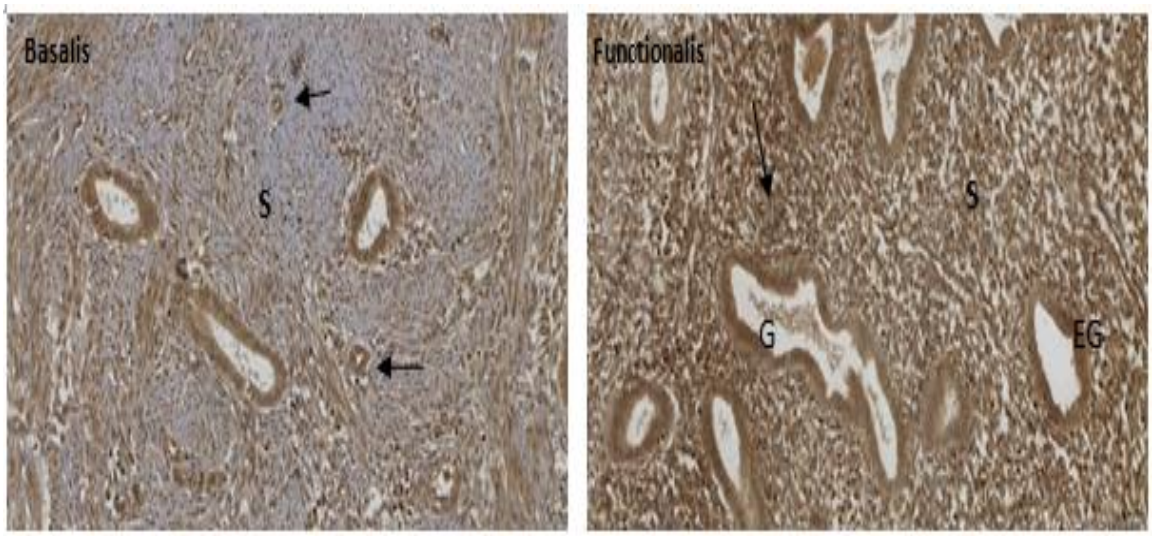


Figure 6.14: Immunostaining reactivity of PGE2 in endometrial layers (basalis and functionalis) for epithelial glandular (EG), stromal (S), intraglandualr (G) and endothelial cells (arrows). The cells in brown immunostaining are positive for PGE2 protein and other cells in blue colour show no PGE2 protein expression.

In women with uterine fibroids, the expression of PGE2 protein was higher during the menstrual phase than the proliferative phase (2.8±0.2 (2-3) & 2.3±0.2 (1-3), $P<0.05$ respectively) but not the secretory phase (2.3±0.2 (2-3) (Figure 6.15). The difference in EP2 receptor mRNA was not statistically significant in endometrium across the menstrual cycle (Figure 6.16).

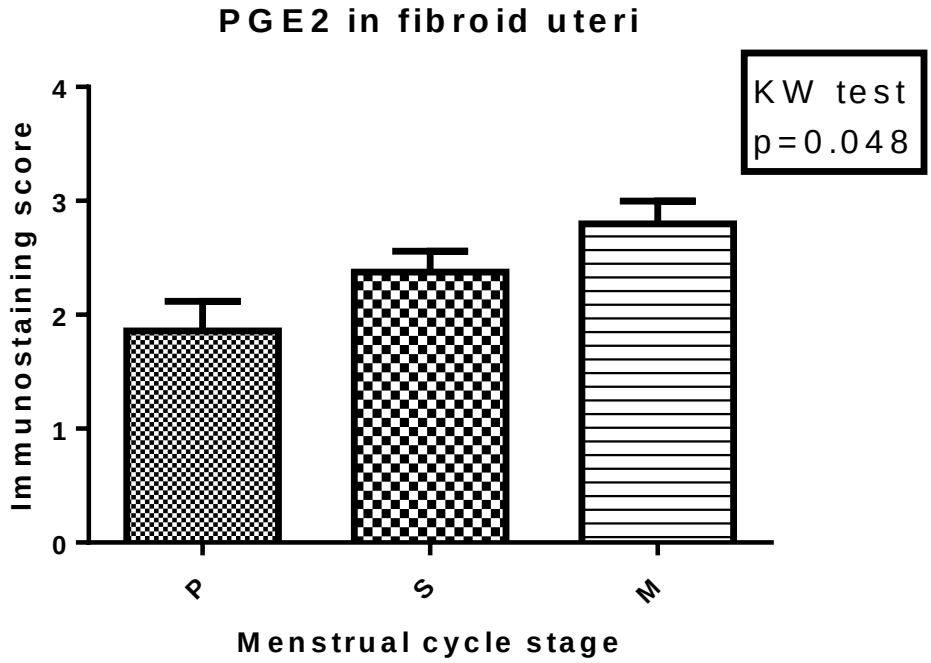


Figure 6.15:Imunostaining intensity level of PGE2 protein in endometrium in women with heavy menstrual bleeding and uterine fibroids across the menstrual cycle. Semiquantitative 4 scale scoring method was used, 1 for mild (<10%), 2 for moderate (10-50%) and 3 for intense staining (>50%).

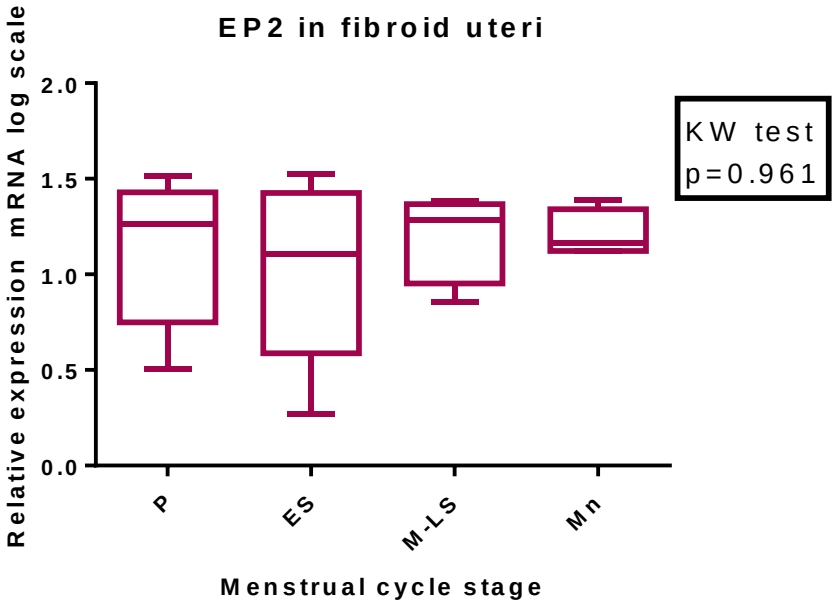


Figure 6.16:Comparison ofEP2 receptor expression levelsin endometrium of women with heavy menstrual bleeding and heavy menstrual bleeding across the menstrual cycle. (Log scale was depicted)

6.3.3.3 Differences between groups

There was no difference in the expression of PGE2 protein in endometrium for women with heavy menstrual bleeding between groups. In the addition, the level of EP2 receptor mRNA was not significantly different between them.

6.3.4 PGE2 and EP2 expression in myometrium and fibroid tissue

The immunohistochemistry results show higher infiltration of PGE2 protein in fibroid tissue in comparison with myometrium, where in the later PGE2 mainly infiltrating into endothelial cells and around blood vessels (Figure 17).

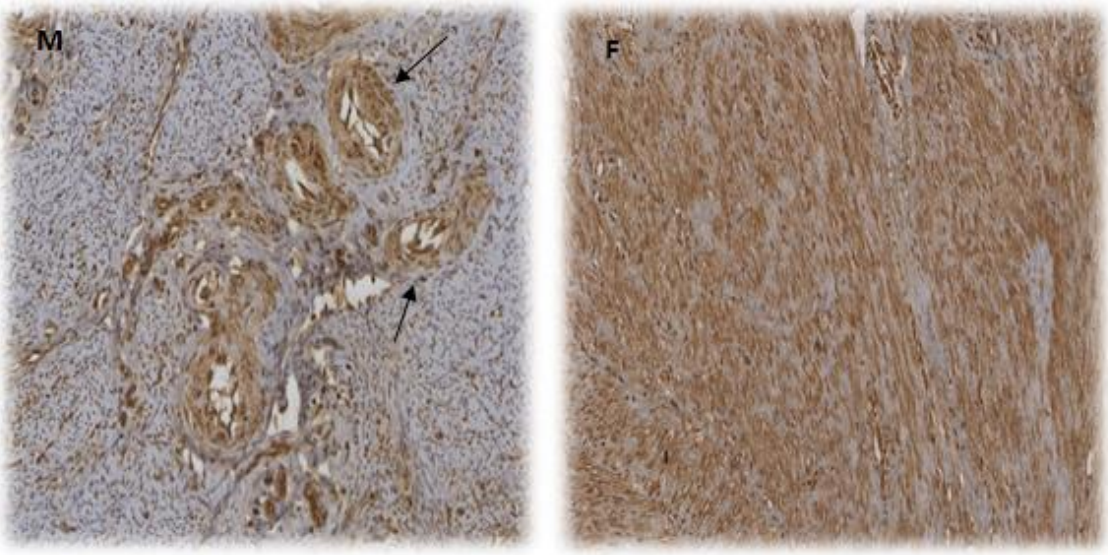


Figure 6.17: The expression of PGE2 protein in myometrium (M) and uterine fibroid (F) in women with uterine fibroids. Black arrows point to blood vessels in myometrium. The cells in brown immunostaining are positive for PGE2 protein and other cells in blue colour show no PGE2 protein expression.

6.4 Discussion

The data presented in this study identify the expression and localisation of COX-2 and PGE2 in stromal, epithelial, and endothelial cells of the human endometrium across the menstrual cycle in women complaining of heavy menstrual bleeding with uterine fibroids and with no uterine pathologies. However, the expression of COX-2 was predominantly in glandular epithelial cells,whereas the expression of PGE2 was high in endometrial stromal and epithelial cells. Our results show over-expression of COX-2 and PGE2 synthesis in endometrium across the menstrual cycle, although COX-2 expression and PGE2 up regulation was mainly during the mid-secretory and the menstrual phases for both groups. This was partly in agreement with previous studies [411-413], which demonstrated upregulation of COX-2 expression and PGE2 synthesis during the menstrual and the proliferative phase. However, the demography of studied subjects was different. The COX-1 mRNA was high in endometrium for women with heavy menstrual bleeding and this may indicate a role of COX-1 over – expression in the pathophiology process of heavy menstrual bleeding.

In general, the premenopausal human endometrium undergoes phases of proliferation and apoptosis during successive menstrual cycles. These phases are observed largely in the functionalis layer of the endometrium, which is shed at menstruation before regenerating during the proliferative phase of the subsequent menstrual cycle. Over-expression of COX enzymes and enhanced synthesis of PGE2 may be due to dysregulation of prostanoids in endometrium, which may cause menstrual abnormality. In heavy menstrual bleeding, stromal expression of COX-2 and PGE2 are predominantly localized in the functionalis layer of the endometrium and extend to the basalis and myometrial regions. Although previous studies suggest the crucial role of PGE2 in human endometrium particularly in cellular mitogenesis and survival, the exact role has not been fully clarified. On the other hand, over expression of COX-2 and enhanced synthesis of PGE2 have been shown to promote the proliferation and survival of cells in colon epithelial cells [405, 414] through up-regulated expression of antiapoptotic genes such as bcl-2 [414]. Therefore, in human

endometrium, it is reasonable that PGE2 may be activating similar mechanisms that promote glandular epithelial cell proliferation and/or survival. This is supported by recent data confirming over expression of bcl-2 endometrial glandular epithelial cells [415, 416].

PGE2 function in glandular epithelial cells may also be associated with the regulation of uterine angiogenesis. Over expression of COX-2 and increased production of PGE2 in epithelial cells have been associated with the expression of angiogenic factors, such as vascular endothelial growth factor [417]. In the human endometrium, vascular endothelial growth factor expression is localized to the glandular epithelial cells throughout the menstrual cycle [318, 418], and PGE2 has been shown previously to up-regulate the expression of vascular endothelial growth factor in a number of different cell types [419]. In women with uterine fibroids, the expression of COX-2 and PGE2 synthesis upregulated in myometrium and the expression of PGE2 were high in perivascular and stromal cells but the expression of COX-2 was almost confined to perivascular tissue. Higher expression of both COX-2 and PGE2 synthesis is found in fibroid tissue than myometrium which reflects upregulation of COX-2 expression and PGE2 synthesis in fibroblast cells. This may lead to up-regulation in the expression of vascular endothelial growth factor in fibroid tissue and thus enhanced fibroid development and growth.

The measured EP2 receptor mRNA demonstrated high expression of EP2 receptor across the menstrual cycle particularly in the mid- late secretory and menstrual phase, which suggests an important role for PGE2 in heavy menstruating endometrium during these phases. In addition, the expression of PGE2 was associated with a high level of EP2 receptor mRNA ,but this was inconsistent with a report by Milne et al.[401]. PGE2 induces vasodilatation via perivascular EP2/EP4 receptor[388], whereas activation of EP receptors on endothelial cells may regulate angiogenesis. Endometrial angiogenesis takes place throughout the menstrual cycle, in the functionalis phase rather than the basalis [420]. Recently, COX-2 and PGE2 have been linked directly with endothelial cell function and angiogenesis[406]. Hence, treatment of endothelial cells with selective COX-2 inhibitors has been shown to reduce microvascular tube formation, and this can partly reverse angiogenic effect by means of PGE2 [407].

Using non-steroidal anti-inflammatory drugs, which inhibit PG synthesis, as treatment of choice to reduce excessive menstrual blood loss in women reporting heavy menstrual bleeding, is a further evidence of a role for PGE2 in uterine vascular [421]. In addition, increased receptor expression, has been observed in the uteri of women with heavy menstrual bleeding [422] and the use of non-steroidal anti-inflammatory drugs to reduce menstrual blood loss also reduces PGE2-binding sites within the uterus [104], all this confirm the role of COX-2,PGE2 and EP2 receptor in menstrual abnormality.

6.5 Conclusion

It can be concluded that over-expression of COX 2 and PGE2 synthesis in endometrium in women with heavy menstrual bleeding reflect their crucial role in menstrual abnormality. Additionally, COX-1 suggested having a role in the mechanism of heavy menstruation as well. The upregulation of COX-2 expression, PGE2 synthesis and EP2 receptor to high levels in myometrium and uterine fibroids, may indicate their role in development and/or growth of uterine fibroids.

Chapter 7

7. The expression of IL8 in endometrium during menstruation

7.1 Introduction

Interleukin-8 (IL-8) is a proinflammatory cytokine associated with neutrophil chemotactic/activating and T cell chemotactic activity[68]. IL8 acts via two G protein- coupled receptors (CXCR1 and CXCR2), which initiate various signalling cascades [66]. IL8 has a role in promoting angiogenesis (chemotaxis of endothelial cells)[71], mitogenesis in cancer [423] as well as vascular smooth muscle cells[424]. In addition, increased expression of IL-8 and/or its receptors has been characterised in endothelial cells, infiltrating neutrophils, and tumour-associated macrophages, suggesting that IL-8 may act as an important regulatory factor for tumour growth [73]. Different types of cells can produce IL8 such as peripheral blood monocytes [72], endothelial cells[425], neutrophils[426], cells derived from human choriodecidual cells [427], fibroblasts, and keratinocytes [428].

In human endometrium, IL8 is mainly localised in glandular epithelial cells, which indicates that it may have other functions besides the recruitment of maternal leukocytes. In addition, it was demonstrated in endometrium across the menstrual cycle with upregulation during the late secretory phase[429] and menstrual phase[430]. In endometrium, IL8 is able to activate macrophages and recruit neutrophils and T cells. On the other hand it can act as an autocrine growth factor to promote angiogenesis in endometrial blood vessels, proliferation of endometrial stromal cells and it also facilitates tissue remodelling [431-433].

During normal menstruation, inflammation is an important event [79] and infiltration of inflammatory leukocytes is suggested to be essential for tissue breakdown and remodelling[434]. However, upregulation of this inflammatory process in endometrium can cause several pathological condition such as heavy menstrual bleeding [97]. Inflammation can be controlled by endogenous anti-inflammatory mediators such as sex hormones[435], cytokine interleukin 10 [436], protein annexin A1[437],lipid mediators involving lipoxin A4[438], resolvin D2 [439]and protectins[440]. A delicate balance between pro-inflammatory and anti-inflammatory mediators is essential for controlling inflammation which occurs during physiological events throughout the female reproductive tract.

Before the onset of menstruation, leukocytes influx into the endometrium by enhanced vascular permeability and assistance of chemokines and cytokines. As IL8 is suggested to play an important role in influx of leukocytes into endometrium, the upregulation of IL8 may cause high infiltration and transmigration of inflammatory leukocytes in endometrium. As a result, it may cause dysregulation in breakdown and remodelling process and lead to heavy menstrual blood loss.

IL8 has been identified to be highly expressed in uterine fibroids [441, 442]and adjacent myometrium [442]. These observations bring a concept that IL8 may play a potential role in the growth of uterine fibroids. In addition, IL8 may have a profound biological and clinical significance, specifically in women with symptomatic uterine fibroids having abnormal bleeding.

Pre-operative GnRH agonists administration lead to regression of uterine fibroids and it used by many gynaecologists in the clinical field to decrease their size preoperatively [381, 443]. Administration of GnRH agonists causes acute reduction in the circulating oestrogen level, which might influence alterations in cytokines and chemokines as well as modifications in vascular inflammation. However, these changes in cytokines and chemokines due to administration of GnRH agonists have not been fully clarified.

7.2 Material and methods

7.2.1 Tissue collection

The collected tissue was discussed in details in section 2.2.1.1.

7.2.2 Real time PCR

RNA was extracted with TRI-reagent (Ambion, Life technologies, Paisley, Uk) following manufacturer’s guidelines using phase lock tubes (Microcentrifuge, Greiner bio-one GmbH, Stone House- England, UK). RNA samples were reverse transcribed using MultiScribe (Invetrogen, Life technologies, Paisley, UK) primed

with random hexamers according to the manufacturer's instructions (see section 2.5.4). When there was not enough fresh endometrial samples , RNA was extracted from formalin –fixed paraffin- embedded samples using Ambien kit 1975 (Recover All total nucleic acid isolation kit-Life technology, paisley, UK) according to the manufacturer's instructions (see section 2.5.1.2). Quality of RNA was assessed using an automated spectrophotometer RNA 6000 Nanodrop (Lab tech.com, Ringmer, East Sussex,UK), only half of the RNA samples were assisted by the Agilent 2100 Bioanalyser system in combination with RNA 6000nano chips (see details in section2.5.2).Once the RNA was extracted and quantified, the PCR reactions were carried out using an ABI Prism 7900 (Applied Biosystems) as previously described in section 2.5.6 using duplicate samples. A no reverse transcriptase (containing water) was used as template control. Pre-validated primers and probes were purchased for IL8 (Life technology, Paisley, UK).

7.2.3 **Immunohistochemistry**

The principle method for immunohistochemistry was followed to express the IL8 gene in uterine tissue. The used antibody was IL8 at dilution of 1:50 from R&D (cat. no. AF-208 NA). Retrieve antigen was performed by microwaving sections in pressure cookers in citrate buffer pH 6.0. Vector biotinylated anti-goat (1:200 dilutions) was used as secondary antibody. Sections were washed throughout the method by PBS with adding 0.1% saponin solution.

Immunostaining was assessed in a semi-quantitative manner on a 4-point scale 0=no immunostaining, 1= mild immunostaining (< 10%), 2= moderate immunostaining (10-50%), and 3=intense (>50%). Double scoring by the same observer with 4 weeks interval was performed. Coefficient correlation between manual and automated scoring manners and between double scoring methods wasaccepted at ≥ 7.

7.2.4 **Enzyme-linked immunosorbent assay (ELISA)**

The quantitative concentration of interleukin-8 (IL8) in serum was measured by ELISA. The component required for this procedure was supplied in the quantikine

kit (R&D Systems Europe, Ltd, Abingdon, UK).the quantitative immunoenzymometric sandwich technique was followed according to the manufacturer's instructions.

7.3 Results

7.3.1 The expression of endometrial interleukin 8 protein in heavy menstrual bleeding

Immunohistochemistry staining successively shows the expression of IL8 protein in the stromal and glandular compartment of endometrium in women with heavy menstrual bleeding and in surface epithelial across the menstrual cycle (Figure 7.1).

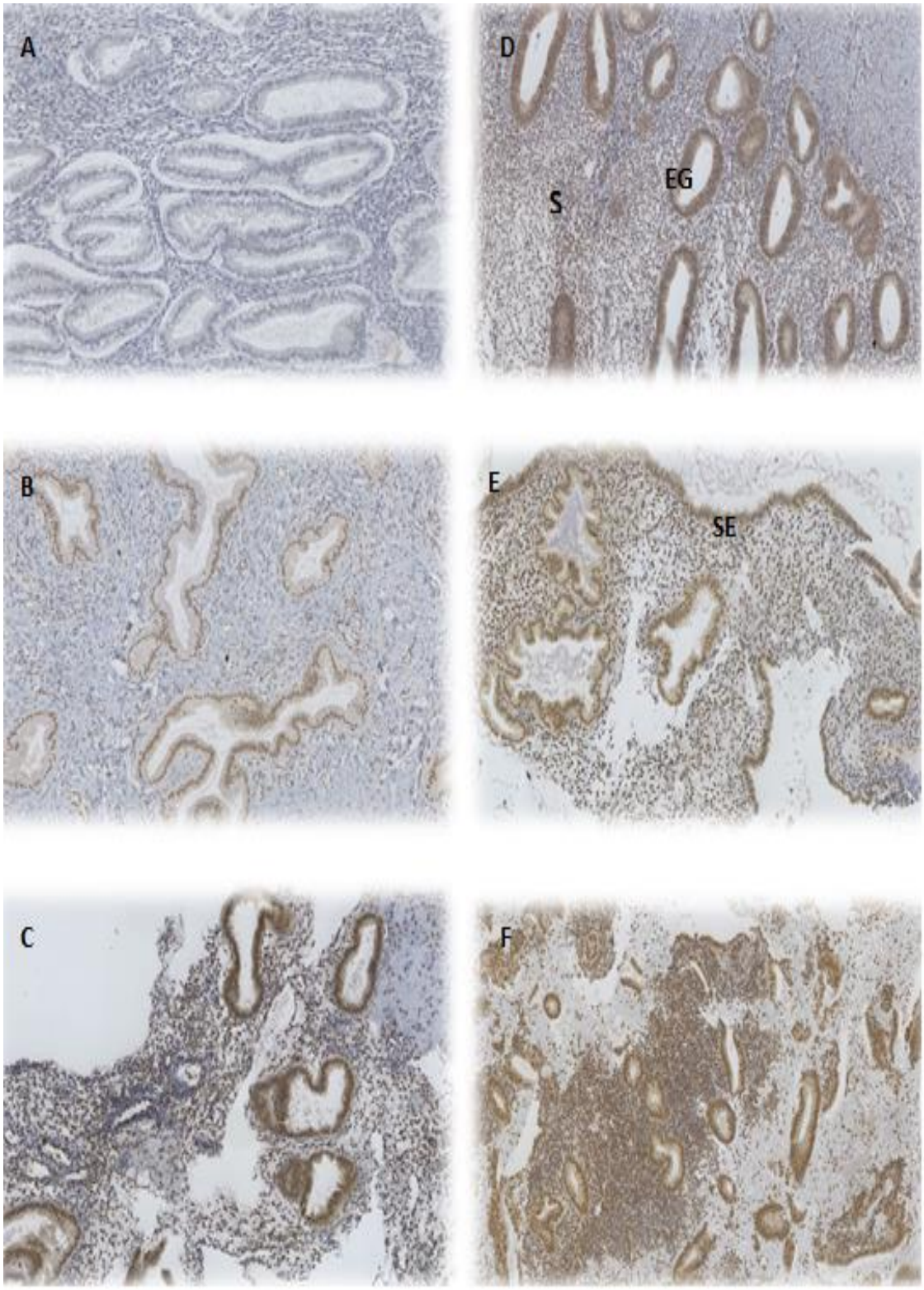


Figure 7.1: The expression of IL8 protein in endometrial stromal (S), epithelial glandular (EG) and epithelial surface (ES) cells across the menstrual cycle; proliferative (D), secretory (early (B), Mid (E), late (C)) and menstrual phase (F). IL8 infiltration was noted in glandular and surface epithelial cells in proliferative and secretory phase, but it is highly expressed in both stromal and epithelial cells during the menstrual phase. The cells in brown immunostaining are positive for IL8 protein and other cells in blue colour show no IL8 protein expression.

7.3.1.1 In women with no uterine pathology

Immunohistochemistry staining shows mild expression of IL8 protein in endometrial stromal, epithelial glandular and perivascular cells during the proliferative and secretory phase, although it is increased during menstruation. In endometrial stroma IL8 infiltration was higher in the menstrual phase than the proliferative and secretory phase ((3±0.0, 1.2±0.2 and 1.2±0.1 respectively), P<0.05) (Figure 7.2).

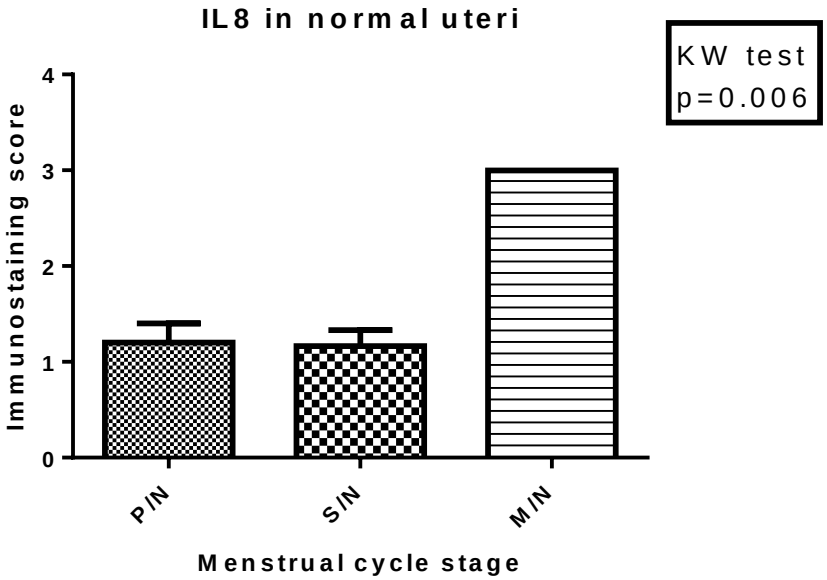


Figure 7.2: The expression of IL8 protein across the menstrual cycle in heavy menstrual bleeding. Semiquantitative 4 scale scoring method was used, 1 for mild (<10%), 2 for moderate (10-50%) and 3 for intense staining (>50%).

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7.3.1.2 In women with uterine fibroids

immunostaining intensity of IL8 in endometrium for women with HMB and uterine fibroids during the menstrual phase was greater than the proliferative and secretory phase (3±0.0, 1.4±0.4 and 1.6±0.2 respectively, P<0.05) (Figure 7.3).

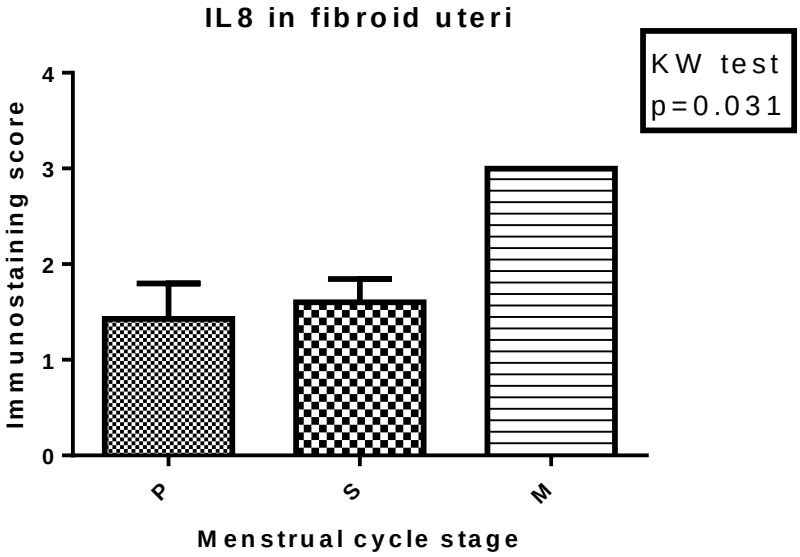

Figure 7.3:The expression of IL8protein across the menstrual cycle in heavy menstrual bleeding. Semiquantitative 4 scale scoring method was used, 1 for mild (<10%), 2 for moderate (10-50%) and 3 for intense staining (>50%).

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Our results found no difference in the expression of IL8 protein in endometrium for women with idiopathic HMB and women with uterine fibroids across the menstrual cycle.

7.3.2 The expression of endometrial IL8 mRNA in women with HMB

7.3.2.1 In women with normal uteri

RT-PCR data showed marked upregulation of IL8 mRNA in the late -secretory and menstrual phase in comparison to proliferative and secretory phases ($p<0.0001$) (Figure 7.4).

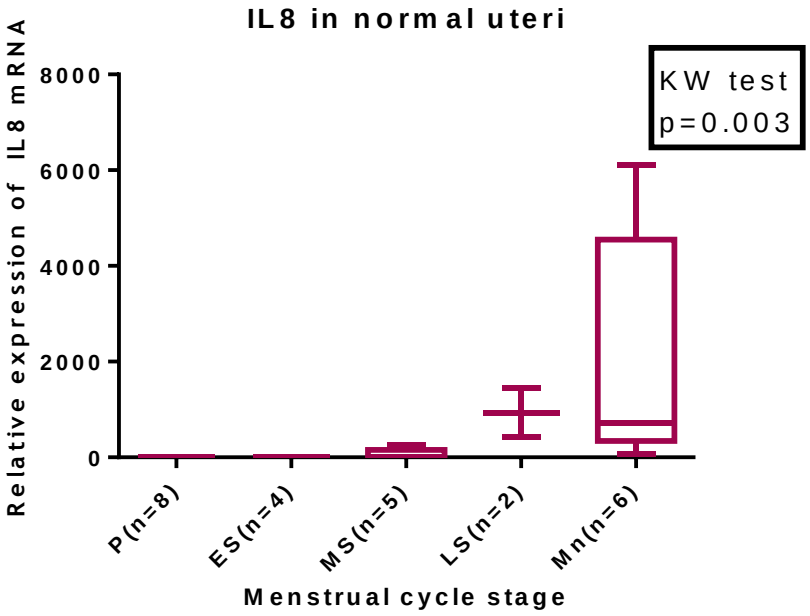

Figure 7.4: the endometrial IL8 mRNA level across the menstrual cycle in women with idiopathic HMB. Kruskal Wallis test was used (KW).

7.3.2.2 In women with uterine fibroids

The measured IL8 mRNA showed a higher expression of the marker in endometrium during the late secretory and menstrual phases than other phases ($p <0.001$) (Figure 7.5).

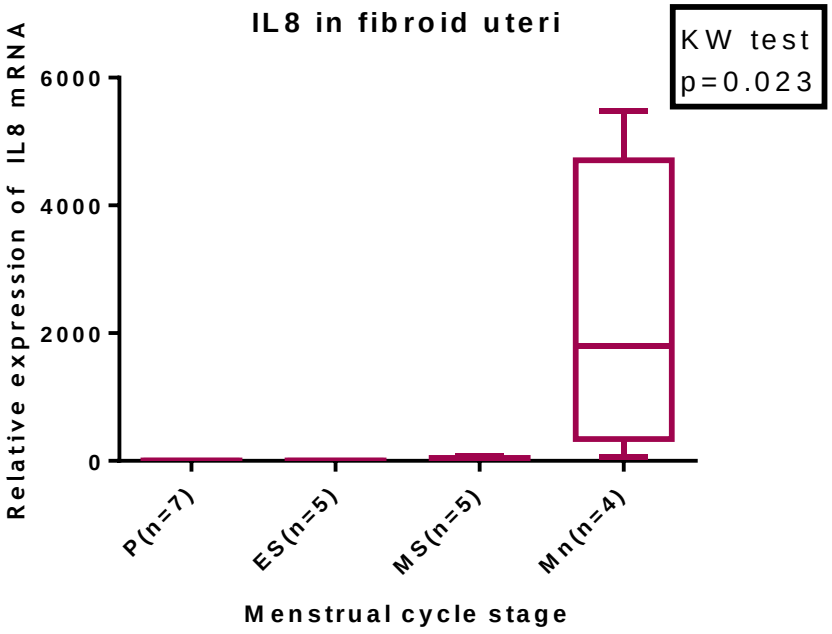

Figure 7.5: The endometrial IL8 mRNA level across the menstrual cycle in women with HMB and uterine fibroids. Kruskal Wallis test was used (KW).

7.3.3 **The expression of IL8 in uterine fibroid**

7.3.3.1 In women with no hormonal therapy

The expression of IL8 protein in myometrium was determined in endothelial cells and minimal expression was found in stromal cells. On the other hand, there was over-expression of IL8 protein in uterine fibroids (Figure 7.6)

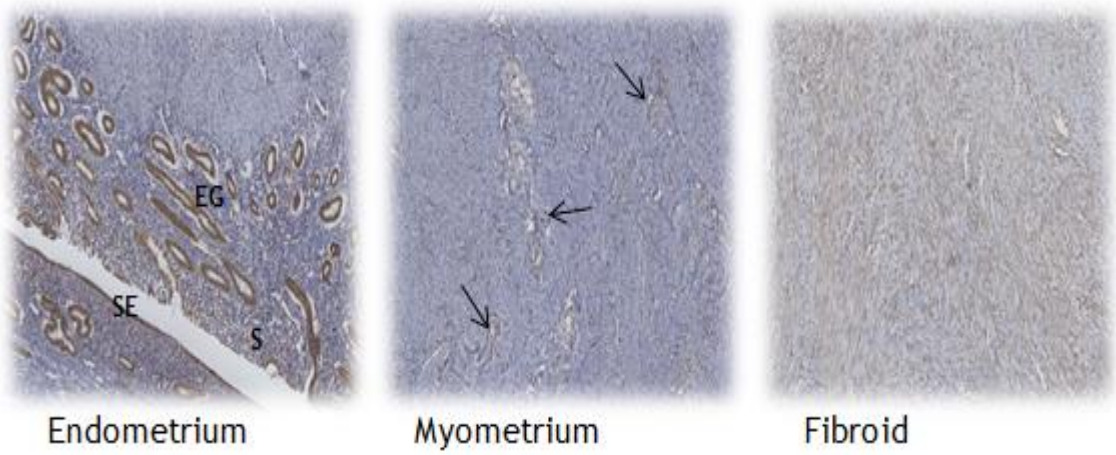

Figure 7.6: The expression of IL8 protein in uterine tissue for women with uterine fibroids. (SE) surface epithelium, (S) endometrial stromal cells, (EG) pithelial glandular cells, and perivascular (black arrow). The cells in brown immunostaining are positive for IL8 protein and other cells in blue colour show no IL8 protein expression.

7.3.3.2 In women who had received GnRH

The immunostaining results show excessive reduction in the expression of IL8 in uterine tissue in women who had received GnRH agonists before hysterectomy in comparison with the expression of IL8 in uterine tissue for women who had received no hormonal therapy (Figure 7.7). The expression of IL8 mRNA was very low, in that it was undetermined after 40 cycles by RT-PCR.

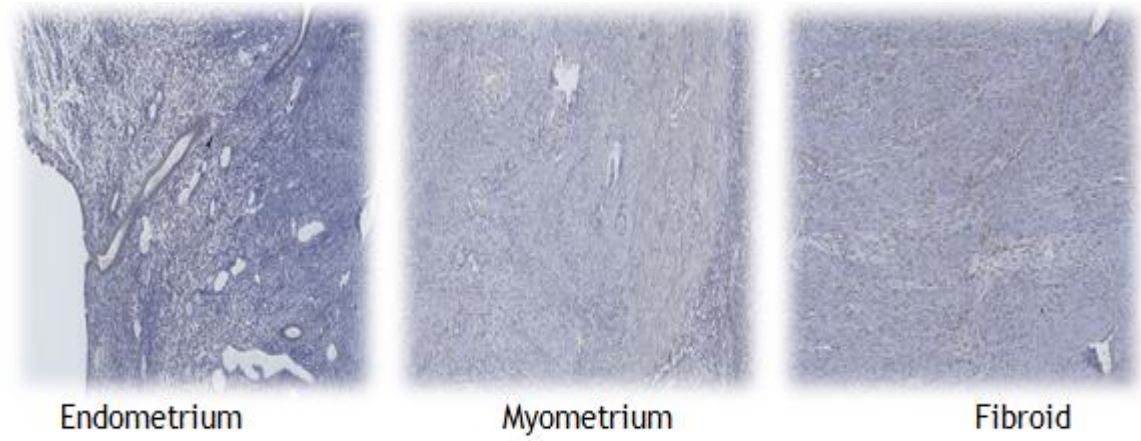

Figure 7.7: A reduction in the expression of IL8 protein in endometrium, myometrium and fibroid tissue was noted in women who had received GnRH agonists. The cells in brown immunostaining are positive for IL8 protein and other cells in blue colour show no IL8 protein expression.

7.3.4 **Serum level of IL8 in women with heavy menstrual bleeding**

There was no difference in the level of IL8 in serum throughout the menstrual cycle for women with idiopathic HMB and heavy menstrual bleeding with uterine fibroids (Table 7.1). However, IL8 level in serum was higher in women who had received GnRH agonists before hysterectomy than those who had received no hormonal therapy ($P < 0.001$) (Figure 7.8).

Table 7.1: The level of IL8 in serum for women with complaint of heavy menstrual bleeding

	IL8 concentration in serum (pg/ml),median(range)			
	In women with fibroid uterus		In women normal uteri	
Menstrual	N= 5	13.2 (8.4-23.63)	N=6	17.25 (10.19-38.48)
Proliferative	N=10	12.05 (8.18-19.21)	N=8	11.52 (8.08-18.96)
Secretory	N=8	11.96 (6.86-23.04)	N=10	9.75 (5.64-26.37)
In GnRH	N=3	35.35 (28.17-62.7)		

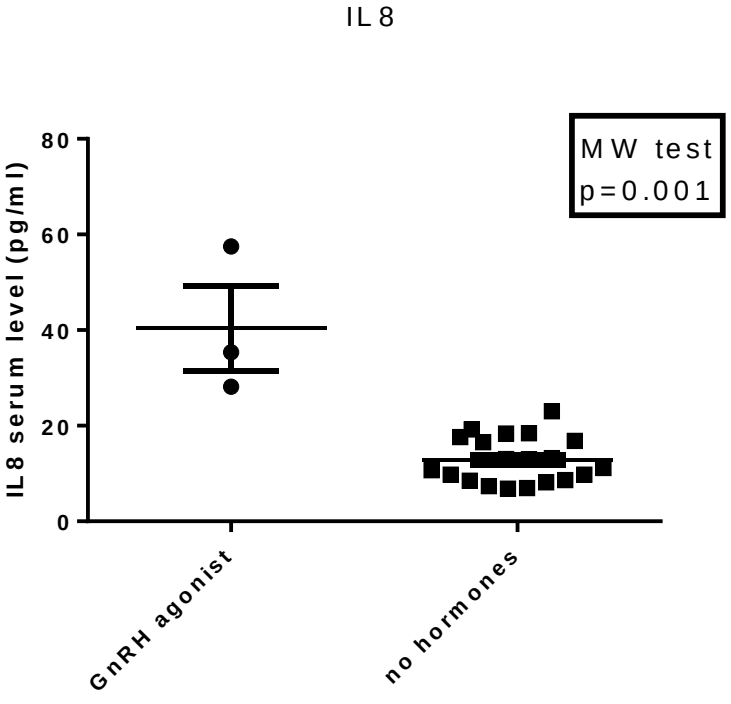

Figure 7.8: The difference in IL8 serum level between women had GnRH agonists and women had no hormonal preparations three months before the biopsy taken. Mann-Witney test was used

7.4 Discussion

Although the precise role of cytokines in heavy menstrual bleeding is not completely known, a compelling body of evidence suggests an inflammatory basis for the physiology of menstruation. Menstruation occurs in women of reproductive age, and the expression of cytokines such as IL-8 in the endometrium fluctuates in a predicTable manner throughout the menstrual cycle [429]. Endocrine factors are involved in modulation of the tissue-specific changes in endometrium and the expression of chemotactic factors that are involved in the influx of the leukocytes during the menstrual cycle. Other report suggests that sex steroids regulate chemokine expression differently in endometrium a cross the menstrual cycle[444]. Endothelial cells play a role in leukocyte extravasation into the endometrium and in the regulation of the tissue-specific changes associated with the leukocyte migration. By mean, the endothelial cell layer acts as the porter of the endometrium for immune cells.

Using immunohistochemistry, it can be confirmed that IL-8 is expressed in all endometrial compartments in women with HMB. The antibody used in this study revealed a significant increase in IL-8 staining in endometrial stromal cells, without significant difference between epithelial and stromal cells. It was observed that there was a significant increase in the IL-8 immunostaining of stromal cells in endometrium during the menstrual phase compared with proliferative and secretory phase. This result indicates that in endometrium from women with heavy menstrual bleeding endometrium , IL8 is mainly upregulated in the stromal compartment as has been demonstrated in previous studies although these have reported IL8 expression in glandular and surface epithelial but not in endometrial stroma [445] . However, those studies investigated the expression of IL8 in women with no heavy menstrual bleeding. This suggests that upregulation of IL8 in stromal cells may contribute to HMB. IL8 is a potent chemokine, and it is reported to control the migration and activation of leukocytes during menstruation. Therefore, overexpression of IL8 may potentiate the chemotaxis and activation action of IL8 and lead to inflammatory cells accumulation in the endometrium, which is a key event in menstruation.

We observed that IL-8 mRNA increase markedly in endometrium of women with heavy menstrual bleeding during the late secretory and the menstrual phase. This was in consistent with other reports that demonstrated increased IL8 premenstrually [27, 399, 429, 430, 446], but we did not observe a significant difference in IL-8 immunoreactivity in late secretory phase from the same group of patients. One possible explanation is that even though IL-8 protein production is highly expressed in women with HMB during the late secretory phase, only two samples were collected during this phase. Therefore, it was not possible to show a difference. However, the study proposes that the increase in IL-8 expression in endometrium of women in the reproductive age, particularly during the menstrual phase may contribute to the pathophysiology of heavy menstrual blood loss. As a result, the increase in IL-8 expression may potentiate leukocyte extravasation and migration into the endometrial tissue under the effects of steroids. This supports the hypothesis that IL8 upregulated when circulating leukocytes influx into endometrium before menstruation as an essential step for tissue breakdown and remodelling.

The study of the women with uterine fibroids, found that the expression pattern of endometrial IL8 was not different from women with no fibroids. This study demonstrated a reduction in the infiltration of IL8 in endometrium to myometrium tissue. However, IL8 was abundant in fibroid tissue, and this is consistent with a recent report [441]which may indicate a role of IL8 in the growth of uterine fibroids particularly since it is known that fibroblasts have the ability to produce IL8[428].

GnRH-agonistisan effective treatment formany gynaecologic disorders. In patients with uterine fibroids, the administration of GnRH-agonists is associated with a significant decrease in uterine fibroid volume and with an improvement in fibroid-related symptoms. These effects are reversible at the end of the therapy, which is therefore considered as a preoperative treatment.

This study found that the expression of IL8 mRNA and protein productionwas reduced in endometrium, myometrium and fibroid tissue in women pre-treated with GnRH agonists. This has been reported before in myometrium and fibroid by Senturk et al. [442].Therefore, it is suggested that inhibition or reduction of IL8 production in endometrium, prevents recruitment and migration of leukocytes

into endometrium, and this maybe one of the mechanisms by which GnRH affects menstruation. Also, reducing the number of immune cells recruited into the endometrium lead to a decrease in their secretory products (cytokines and chemokines) and as a result, this allows the anti-inflammatory mediators to prevent menstruation. In addition, suppressing the production of IL8 in uterine fibroids may be one of mechanisms forthe reduction in fibroid growth. However, the level of IL8 in blood was higher in this group of women as has been previously reported [447].It may be hypothesized that GnRH agonists may promote the production of pro-inflammatory mediators in blood and suggest the induction of inflammation in vascular endothelial cells. Hence, this provokes the detrimental effect of use of GnRH agonists such as hot flashes, where it is previously reported that serum IL8 concentration in women with hot flashes was higher than that in women without hot flashes[448].

7.5 Conclusion

It would be concluded that IL8 has a vital role in heavy menstrual blood loss. In addition it plays a role in the growth of uterine fibroids. Reduction in the IL8 production may be one of the mechanism by which GnRH agonists reduce the size of uterine fibroids.

Chapter 8

8. Discussion

8.1 General discussion

Heavy menstrual bleeding is the main menstrual disorder affecting women in their reproductive years, which places a considerable burden on general practice and secondary health service resources. In addition, it is the most common indication for hysterectomy [87]. It has been suggested that changes in the life style may contribute to this medical disorder, especially in communities where women would experience a greater number of menstrual cycles due to reduction in family size and incidence of lactational amenorrhoea compared to women with larger family size and lactating women.

Although the mechanism of the physiological aspects of the menstrual cycle has been well established, the exact mechanism of heavy menstrual cycle is not fully clear. However, it has become clear that complex cellular and molecular systems are involved in the mechanism of abnormal menstrual bleeding [498].

The molecular mechanism of menstruation in response to progesterone withdrawal is characterised by complex events, which in part involves the production of prostaglandins that are vasocontrictors and lead to reduction in the blood flow to the endometrium. This is following by increased expression of locally acting mediators such as cytokines, angiogenic factors, protease enzymes and further prostaglandins. All these changes together with influx of leukocytes into the endometrium comprise the process of menstruation.

Many previous investigations have supported the role of local disturbance of prostaglandin synthesis, secretion and metabolism in several endometrial pathologies, particularly those that are associated with disturbance in menstrual bleeding and painful menstruation. In addition, a role for COX enzymes and prostaglandins has been determined in reproductive tract pathology such as heavy menstrual bleeding, endometriosis and cancer [96, 387, 499]. This understanding has lead to the introduction of the use of non-steroid anti-inflammatory drugs as treatment for such complaints. Non-selective NSAID and selective COX-2 inhibitors, however, have been associated with gastrointestinal, renal and cardiovascular problems, which highlight the need for further

understanding of COX which is the rate limiting enzyme in prostanoid production [500, 501].

Under normal circumstances, it has been suggested that the responsible cells for prostaglandin production, constitutively express COX-1 and are induced to express COX-2 in response to external stimulation. In heavy menstrual bleeding, significant elevation in the level of mRNA expression of both COX-1 and COX-2 has been demonstrated [398]. As a consequence an increase in the level of the rate limiting enzymes of prostaglandins production in women with heavy menstrual bleeding leads, to the upregulation of the prostaglandins production and signalling. This present study demonstrates significantly increased levels in mRNA expression of both COX-1 and COX-2 in endometrium during the secretory phase, and the expression of COX-2 protein is mainly upregulated at the mid-secretory phase. The established role of COX enzymes in the regulation of angiogenic factors, the over-expression of Cox-enzymes during the secretory phase maybe implicated in the disturbance of endometrial angiogenesis in heavy menstrual blood loss through disturbing in the balance of these factors. These angiogenic alternations may affect vascular development, composition and structural integrity or permeability. Reduction in the control of blood volume loss is one of the mechanisms of heavy menstrual blood loss [149].

Eventually, vascular permeability and leakage at the time of menstruation will be determined by the ratio of pro-and anti-angiogenic factors. COX-2 enzyme is thought to have the ability to augment the expression of VEGF (angiogenic factor) via hypoxia induced factor1 (HIF1), therefore, the over-expression of COX-2 enzyme in HMB would suggest up-regulation of the endometrial VEGFA. The over-expression of both VEGFA mRNA and protein during mid-late secretory and menstrual phase in endometrium obtained from women with HMB in this study supports this suggestion. On the other hand, the over-expression of VEGF can up-regulate the expression of COX-2 in several WAYS. In addition to upregulation in the expression of angiogenic factors, COX enzymes may promote the action of angiogenic factors in HMB by decreasing anti-angiogenic factors, where a reduction in expression of cathepsin D mRNA and protein has been demonstrated in endometrial epithelial cells over-expressing COX-2 enzyme [502].

The remodelling of endometrium during the menstrual cycle requires tight control of angiogenesis factors to coordinate the growth of new vessel formation. Thus, aberration in angiogenic factors would dysregulate the neoangiogenesis process and affect endometrial repair. The VEGF expression in endometrium is upregulated largely during the menstruation in normal cycles, where the endometrial repair process starts in day 2 of the cycle. This may indicate to a role of VEGF in the endometrial repair process. Both hypoxia and PGF2α have been found to play a key role in regulating the VEGF expression. Hypoxia acts via the HIF-1α to upregulate the expression of VEGF, whereas the PGF2α acts through HIF-1α independed pathway.

By investigating the angiogenic factor vascular endothelial growth factor in endometrial stromal and endometrial glandular epithelial cells in women with HMB, this thesis demonstrates VEGF-A protein expression in both stromal and glandular endometrial cells and around the endometrial blood vessels. This supports the hypotheses that suggest VEGF regulates epithelial and stromal development in endometrium under the influence of oestrogen and progesterone; thereby it regulates the growth and differentiation of endometrium during the menstrual cycle. This expression is increased throughout the menstrual cycle, but takes place mainly at mid-secretory phase and reaches the highest in the menstrual phase. In addition, The VEGF-A mRNA level has the same pattern of expression as the VEGF-A protein. The importance of VEGF-A in endometrium is representative in promoting neoangiogenesis, which is the key process in regeneration and remodelling of endometrium during the menstrual cycle. Comparing the expression level of VEGF-A in endometrium in women with normal menstrual blood loss and women with HMB fall out with the remit of this thesis. However, in chapter 2, in endometrium of women with PBAC score of less than 100 points, the level of VEGF-A mRNA was higher than those with higher PBAC score (>100). The work of some authors supports this concept and they found out that the endometrial VEGF-A level is lower in women with normal blood loss than in women with HMB[94]. This result hypothesised that because of the role of VEGF-A in new vessels formation that should start couple of days before the end of the menstrual phase to enhance the regeneration and proliferation of endometrium, low VEGF-A levels may lead

to a lack in new vessel formation and this allows disrupted vessels to bleed for a longer time and leads to HMB. This can be a logical explanation, although in this thesis, comparison between endometrial VEGF-A mRNA levels for women with high PBAC score and low PBAC score was regardless of the menstrual phases, moreover, concerns about the process by which pictorial blood loss assessment chart was used and the low sample size gives a possibility of having this result by chance.

The most known role of IL8 as a CXC chemokine is a potent chemoattractant for neutrophils. However, it also has mitogenic properties and an important role in the process of angiogenesis in vivo, indicating that IL8 is a key marker in endometrial repair. The upregulation of IL8 mRNA and protein expression shortly after progesterone withdrawal during the menstrual phase, where the endometrial repair process takes place, support this hypothesis. Both hypoxia and PGE2 upregulate the expression of IL8. The hypoxia acts via the HIF-1α to upregulate IL8 production and PGE2 act through another nuclear transcription factor present in the endometrial tissue in premenstrual time (NF-KB). However, the synergistic up-regulation of IL-8 mRNA observed in endometrial cells exposed to PGE2 and hypoxic conditions simultaneously indicates interaction between HIF-1α and NF-KB in regulating IL-8 expression.

The ratio of PGF2α/PGE2 decreases in endometrium of women with heavy menstrual bleeding due to an increase in the production of PGE2 and decrease in the expression of PGFα2 receptors. As a result, the vasoconstriction of spiral arterioles may reduce and/ or decrease and a hypoxic insult may happen. Hence, the PGE2/hypoxia-induced pathway interaction would be affected, resulting in aberration in IL8 expression, and the endometrial repair process, which may cause heavy menstrual blood loss. In this study, the IL8 protein expression in endometrial stromal, glandular and endothelial cells is highly upregulated during the menstrual phase compared to mild expression during the proliferative and the secretory phase. Whereas, the IL-8 mRNA is upregulated during the late secretory phase and reaches the highest expression during the menstrual phase. According to previous work, the expression of IL8 protein in the normal menstrual cycle is upregulated during menstruation and it is faint during other phases. The expression of IL8 protein in this study for women with

heavy menstrual bleeding indicates upregulation throughout the menstrual cycle and is in keeping with the expression pattern been previously in normal cycles.

Upregulation of IL8 expression is accompanied by upregulation in the influx of neutrophils into the endometrium. In this study, neutrophils showed increased expression in the late secretory phase and menstrual phase compared to faint or absent expression in the rest of the cycle, this suggests that their infiltration into the endometrium is regulated by IL8 and they play a part in endometrial repair.

Both neutrophil and uNK cells have a key role in the angiogensis process, thus dysregulation in the infiltration of these cells may contribute to menstrual abnormality. Some neutrophils show expression of VEGF in other studies, and this supports their function in angiogensis promotion. The uNK has a role in production of angiogenic growth factors similar to VEGF. Although evaluating this hypothesis was not the aim of the present study, the study has a propensity to support this thought as both VEGF and uNK upregulated postovulation, during the mid-secretory phase, late secretory and menstrual phases, and uNK cells show perivascular expression, indicating their role in remodelling of spiral arterioles. Therefore, they have a fundamental and major importance in promotion of angiogenesis. Macrophages contributed to remodelling of arterioles through macrophage-mediated phagocytosis and activate MMP-9. The postovulation expression of macrophages indicates their functional remodelling of arterioles.

The comparative study failed to find a significant difference in the expression of VEGF, PGE2, COX-1, COX-2, and IL8 either protein or mRNA, between endometrial tissue for women with heavy menstrual bleeding and uterine fibroids and women with HMB and normal uteri. This result indicates that these vascular markers contribute to HMB in both women with fibroids and women with normal uteri in the same manner. However, small cohorts in both groups could be a reason for not obtaining a difference between groups, particularly throughout the menstrual phases. In addition, if the nearer site of fibroids from the endometrium would affect more the endometrial physiology, in this study a

considerable number of women were with subserosal fibroids, and the majority of them were with intramural fibroid. Women with any type of uterine fibroids may have HMB, but submucosal fibroids are the type of fibroid most likely associated with menstrual abnormality. It would be valuable to compare samples of each type of fibroid separately with uteri without pathology, but small cohort size when the results were grouped by fibroid position limited this.

The study failed also to find differences between the two groups regarding the endometrial leukocyte infiltration. The factors that caused the limitations discussed above may also play a role in this result as well. The intramural, and subserosal fibroids are not associated with endometrial distortion, hence, they may not upregulate leukocyte influx into the endometrium. In addition, in the present study, the comparison of endometrial leukocytes depends on the relative number of leukocytes in the endometrial stroma in both groups. However, differences between leukocyte in endometrium from women with fibroids and women with normal uteri may be determined by investigating cell activities instead /or besides cells number. The activity of leukocytes can be evaluated by investigating the activity pathway for these cells which are represented in the expression of some activating ad inhibiting receptors, and some cytokines that play a crucial role in activating leukocytes.

This study indicates high expression of VEGF protein in fibroid tissue, which is significantly higher than that in myometrial tissue. The upregulation of VEGF expression seems to be situated in the extracellular matrix and fibroblasts, indicating to a fundamental role of VEGF in the growth and may be the development of uterine fibroids. Fibroid tissue has less vascularity than normal myometrium and they rarely transfer to malignancy. If VEGF is an angiogenic factor and the best known role is a promoting factor of neoangiogensis, the presence of VEGF in fibroid tissue raises a question regarding its role there. The study suggests another unidentified function of VEGF. However, condensation of fibroid tissue which may concentrate the expression of VEGF in the fibroid tissue would be another explanation of this high expression of VEGF in fibroid tissue. In the study, differences in the VEGF mRNA levels between fibroids and myometrial tissue are not significant, which may support the later explanation.

All COX-2, PGE2 and IL8 have higher protein expression in fibroid tissue than in myometrial tissue, indicating their role in growth and may be the development of uterine fibroids. These markers as discussed above have a fundamental role in angiogensis process; their expression in myometrial tissue is concentrated mainly around blood vessels, indicating their effect on endothelial cells. However, their role in fibroids is not fully elicited. On the other hand, their expression in fibroid tissue seems to be influenced by steroid hormones.

This study elicited down regulation of the expression of these local markers in fibroid tissue from women who received GnRH agonists before operation. Hence changes in the steroid hormonal level may dysregulate the expression of these markers in the fibroid tissue. The GnRH is well known as a therapeutic option to reduce the size of fibroid and reduce the blood loss during surgery. The downregulation of these pro-inflammatory markers by using this drug indicates their role in the growth of fibroids and points to the inflammatory pathway as a manner in fibroids expansion. Nevertheless, the relative expression of VEGF mRNA is significantly upregulated in endometrium from women who had GnRH agonists compared with the VEGF mRNA level in endometrium from women who received no hormonal therapy. This increase in the VEGF mRNA level, which was associated with a hypoestrogenic condition, would support the hypothesis that there is a lower level of endometrial VEGF in women with HMB than that from women with normal blood loss. However, for women who received GnRH, the endometrial VEGF protein expression shows downregulation in some areas and high concentration in others. That raises a possible explanation, that cells exposed to GnRH work in a compensatory manner, where some of cells reduce the expression of VEGF another increase the expression of VEGF protein. Women, who receive GnRH, usually have very thin endometrium, and this causes condensation in the endometrial structure, which might be the reason for increase in the level of VEGF mRNA expression. This difference in the VEGF mRNA level is interesting and further evaluation would be valuable as this is the first time this result has been reported. This result may be obtained by chance because of small sample size in this group (3 subjects) but it was very consistent and marked.

The serum level of VEGF shows no difference between women who received GnRH agonists and women who had no any hormonal therapy. On the other hand,

the serum level of IL8 in the group that received hormone was significantly higher than that of the other group. Therefore the study indicates a relation between using GnRH agonists and hot flashes; where the later increase the inflammatory mediators in blood, suggesting induction of inflammation in the vascular endothelial cells.

8.2 Summary

In normal menstrual cycle, progesterone withdrawal results in an upregulation of inflammatory mediators, production of proteases such as MMPs and plasminogen activators, leukocytes influx and expression of VEGF-R2 in endometrial basalis[82].

Hypoxia is coincident with progesterone withdrawal and upregulation of VEGF, which acts via binding to VEGF-R2 in the basalis layer and the shedding of endometrium takes place in the endometrial superficial (functionalis) layer.

Both stromal cells and leukocytes play a role in upregulation of MMPs, which is under a paracrine/autocrine action and a play a fundamental role in extracellular breakdown. The endometrial repair takes place in day 2 of the menstrual cycle and chemokines such as IL8 and IL15 play a key role in this process beside the proangiogenic factors as VEGF. Endometrial shedding and repair is a complex process, including cellular and molecular mechanisms (Figure 10.1).

Aberration in these pathways contributed to menstrual abnormalities. In heavy menstrual bleeding a higher expression of COX-2, PGE2 has been elicited, whereas the level of VEGF needs further investigation.

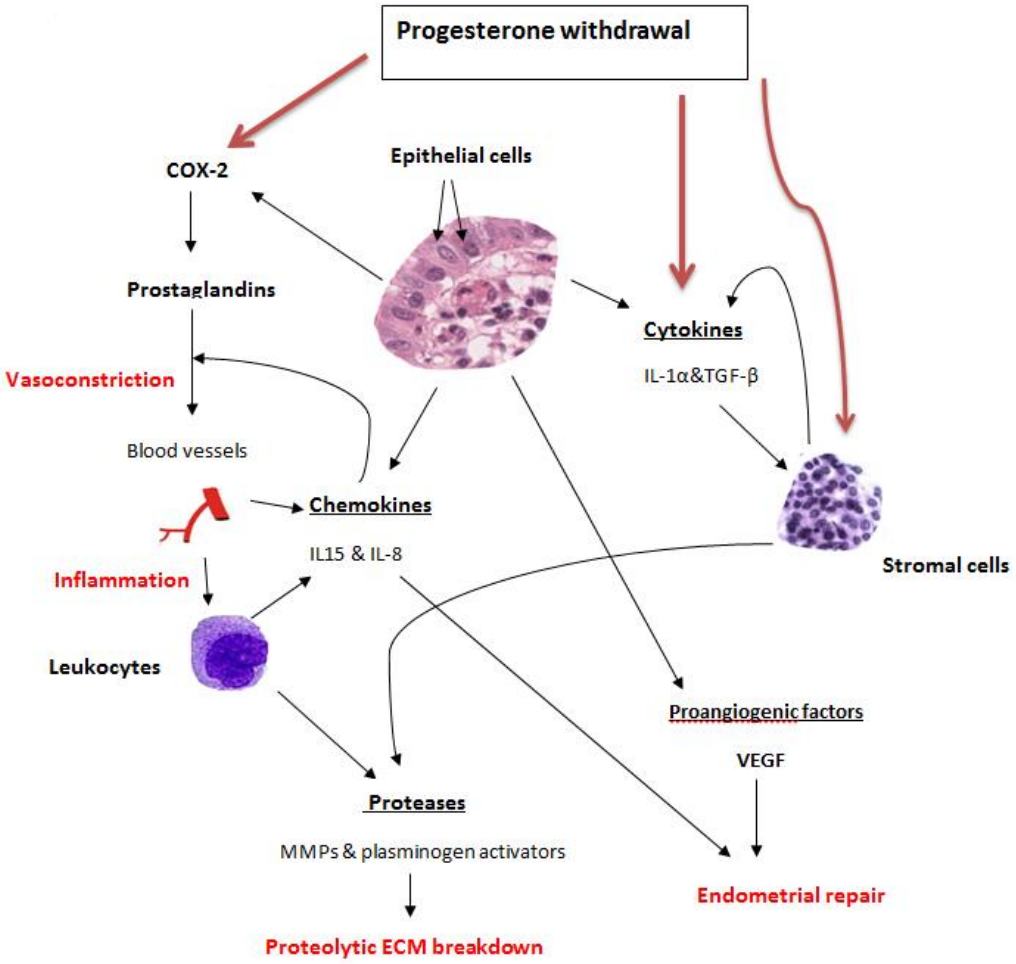

Figure 8.1: Summary of the endocrine and paracrine control of menstruation.

8.3 Work in the future

This interesting work tried to find out the mechanism by which uterine fibroids lead to heavy menstrual bleeding by investigating the expression of VEGF and other cytokines in endometrium for women with HMB and uterine fibroids and compare these data with the levels in endometrium from women with HMB and no uterine pathology. Regardless of the negative results that the comparative study found, which may have been contributed to in part by having small cohorts in each stage of the menstrual cycle, the thesis supports some findings from work undertaken while this study was in progress, but also raised new questions regarding other findings.

The author suggests that upregulation of the studied markers is the end-step contributing to heavy menstrual blood loss in presence of uterine fibroids. This upregulation of the expression of these makers works in the same manner in both uterine fibroid and absence of uterine pathology. The difference may be in the mechanism by which uterine fibroids stimulate the regulatory factors of these local markers. Finding an alternative hypothesis through which fibroids can cause HMB is a challenge for future work. The other challenge in the future is to come to a conclusion regarding the relationship between uterine fibroids and menstrual abnormality, whether it is an association or causation. In simple words, women with susceptibility to uterine fibroids may also have susceptibility to menstrual abnormality. In that there is requirement for knowledge of the gene structure and sequence in the human endometrium across the menstrual cycle, and abnormality of these genes in menstrual disorders. It is also important to enhance our knowledge on the aberration in gene expression in uterine fibroids.

These approaches can provide a great understanding of various genetic and molecular pathways when applied to specific endometrial pathology such as uterine fibroids.

Exploring the global transcriptional and signalling effect of drugs such as SPRMs on the endometrium of women with HMB and uterine fibroids, this offers an understanding of the effect of these drugs and their benefits in presence of uterine fibroids.

In the near future, it would be great if I have the opportunity to help in discovering the mechanism by which uterine artery embolisation affects the

endometrium and reduces the menstrual blood loss, and changes in the expression of local markers after the procedure.

Appendices

Appendix 1

A. Ethical approval
B. Patient information sheets (group1, 2 and group 3)
C. Consent form
D. GP Letter
E. Baseline questionnaire (All groups)
F. Follow up questionnaire (Group 3)

Appendix 2

A. Blood loss assessment chart (PBAC)

Appendix 1

Appendix 2

Presentations

- School of Medicine Launch day, Glasgow (2012). Uterine fibroids and heavy menstrual bleeding: what is the relation and the solution?

- Munro Kerr Society for the study of reproduction,Glasgow (2013), How gynaecologists treat uterine fibroids in the UK and Libya.

- Society for Gynecology Investigation (SGI), Istanbul (2013), The expression of endometrial vascular endothelial growth factor -A and its receptors in idiopathic heavy menstrual bleeding and in the presence of uterine fibroids.

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